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PROCEEDINGS OF

THE ROYAL SOCIETY.

Section B.—Biological Sciences.

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Croonian Lecture.—*The Messages in Sensory Nerve Fibres and their Interpretation.*

By E. D. Adrian, F.R.S., Foulerton Research Professor.

(Lecture delivered June 4, 1931—MS. received June 20, 1931.)

[Plates 1–5.]

The nervous apparatus which intervenes between stimulus and sensation has been the subject of more than one Croonian lecture. It may claim to be a suitable topic for a discourse on the "Causes and reasons of the phenomena of local motion," but it is a dangerous topic as well, since it forces us to consider the mind as well as the body and to attempt the measurement of phenomena which lie outside the framework of the physical sciences. But in spite of its many difficulties the field is one in which mental and material changes are brought into the closest possible relation, and it should be worth exploring if for this reason alone.

The sensory apparatus is most often studied by comparing stimulus and sensation. The method discussed in this lecture is a recent introduction scarcely assimilated to the older lines of work; it deals with an intermediate link in the chain, for it attempts to compare both stimulus and sensation with the messages which pass up the sensory nerve fibres. It has depended on an improvement in the technique of nerve physiology and it is sad to recall that this improvement followed closely on the death of the investigator who was most fitted to profit by it. Nineteen years ago Keith Lucas, then only 33 years of age, delivered the Croonian lecture on the "Process of Excitation in Nerve and Muscle." His lecture is a characteristic example of the rigid analytic method which he used in formulating the problems of nervous activity, but it does not reveal the mastery of experimental technique which enabled him to solve them. Much of his later work dealt with the action currents of nerve, the brief electric changes which are our main clue in the study of nervous conduction. He used the capillary electrometer to record them and his design of the electrometer system and of the machine for analysing its records showed his remarkable gifts on the instrumental side; it is quite certain that our knowledge of all that takes place in the nervous system would have advanced much further by now had he lived to make use of the newer methods of electrical measurement.

The advance in technique is due to the development of the triode valve amplifier. Whereas in the year of Keith Lucas' Croonian lecture it was just possible to make an accurate record of the potential wave produced in a nerve trunk by exciting all its fibres simultaneously, a change of a few millivolts lasting a few thousandths of a second, it is now a simple matter to record the change of a few microvolts due to the independent action of each nerve fibre. Moreover, we are no longer compelled to use a sensitive recording instrument like the capillary electrometer, but are free to select any system which will work rapidly enough—and here I must pay a tribute to my colleague Matthews, who has simplified the technique so much by his development of an oscillograph which is not only very accurate but literally unbreakable as well (Matthews, 1928). The method has opened up two lines of work which are particularly concerned with the sensory nerves. At St. Louis Gasser and Erlanger have taken advantage of the unrestricted choice of recording system and have used the cathode ray oscillograph to analyse the potential changes which follow on electric stimulation, and at Cambridge the enhanced sensitivity has allowed us to study the activity of individual nerve fibres. Gasser and Erlanger's work has stressed the wide variation between different types of fibre; the study of individual fibres began by stressing the common features in their activity, but it has now reached the point where it must take account of the variations as well.

The Nature of the Message Transmitted by Nerve Fibres.

Before discussing our main problem, that of the nervous mechanism responsible for pain, we must consider the general nature of the evidence which can be obtained from these electrical records.

When a motor nerve is stimulated electrically a momentary disturbance or impulse travels down each nerve fibre and causes the muscle to contract.

The impulse is an explosive change of fixed magnitude and duration which spreads along the fibre leaving in its wake a temporary disorganisation or refractory state. Wherever it appears the impulse is associated with a change of potential; the active region in the fibre becomes negative to the inactive regions, and thus the progress of the impulse can be followed by placing the nerve on two electrodes leading to an electrometer and recording the deflections which occur as the active region arrives first at one electrode and then at the other. These deflections are of opposite sign and the interval between them gives the rate of conduction of the impulse. All this had been worked out by the classical methods of nerve physiology, using isolated nerves stimulated by an induction coil, but there was very little doubt that the messages normally transmitted were made up of impulses of the same kind. Direct evidence of this was forthcoming as soon as it became possible to detect the potential waves in each nerve fibre, for it was found that potential waves of the familiar type passed up the sensory fibres when sense organs were stimulated and down the motor fibres when movements were in progress (*cf.* fig. 1, A and B, Plate 1). The "action potentials" in each fibre are of fixed size and duration when they occur at intervals great enough to allow the recovery of the fibre after each impulse, and in fact the impulses which occur under normal conditions are identical with those set up by electric stimulation of the nerve trunk.

There is no doubt that the transmission of messages made up of repeated impulses is an event which is constantly occurring in the peripheral nerve fibres, but are these the only kind of messages which can be transmitted? It can do no harm to leave this question unanswered and to keep a look out for some other form of nervous activity, but as fresh evidence accumulates it becomes increasingly difficult to believe that any other form is possible. In general it may be said that in suitable conditions we find a transmission of impulses, as revealed by their electric effects, whenever we have reason to suppose that a nerve is in action, and that the main features of the impulse discharge agree with what we should expect to find in the nervous message judging from the conditions of stimulation and from the effects which the message produces. To quote a recent example: Dr. Stella has recorded the discharge of impulses in the nerve from the sinus caroticus and finds that it occurs when the artery is expanded by a pressure of 50–80 mm. Hg, and again when the pressure falls to zero. These pressures turn out to be in close agreement with those producing reflex effects on the circulatory system. Many other instances of such agreement could be given.

The association of impulse discharges with nervous activity is not confined to the peripheral nerves of vertebrates, though most of the evidence relates to them. The nerve fibres of arthropods and insects show repeated potential changes of the same kind when sense organs are stimulated and when movements take place. The time relations of each active period are slower than in the medullated fibres of a vertebrate, but there is the same uniformity in the size and shape of the responses. The arrangement of the central nervous system in insects makes it possible to examine the fibres in the nerve cord connecting one ganglion with another, and these, too, show potential waves of the familiar type (fig. 1, C). Thus in insects at least the messages which pass from one part of the central nervous system to another are of the same kind as those in the peripheral nerves.

It would be incorrect to say that there is no evidence for the transmission by nerve fibres of any other change than the impulse, for Lapicque and his collaborators (1928) have found alterations in the chronaxie of motor nerves following immediately on injury to the nerve root or to the central nervous system. The chronaxie gives a measure of the rapidity of the excitatory process, and it is possible that transmitted changes in chronaxie might play some part in determining the effects of sensory stimuli. But a transmitted effect of this sort could scarcely be classed as a nervous message, for it does not arouse the activity which is normally aroused by the nerves. The effect deserves the most thorough investigation, but it need not modify the conclusion that nervous communication is carried out by the discharge of impulses each of which produces the characteristic potential change in the nerve fibre.

Naturally there are cases where we could scarcely expect to record the electric effects, for a small amount of short circuiting by inactive tissue may reduce the potential gradients to a negligible value. To avoid this the nerves must be suspended in the air on the two electrodes which connect them with the recording system, and even so the short circuiting which occurs in the nerve trunk itself makes it difficult to record the potential changes in the smallest fibres. A probable failure of this kind will be dealt with later, but the failures have become fewer as the technique improves.

The general likeness between the messages in different kinds of nerve fibre can be seen from fig. 1 (Plate 1). In a sustained discharge the impulses usually occur with a definite rhythm which varies with the intensity of the excitation, *i.e.*, with the rate of change, the intensity and the duration of the stimulus in the case of a sense organ and with the range and force of the movement in the case of the motor fibres. The production of such discharges must depend on some fairly simple reaction of a kind which can occur in many excitable

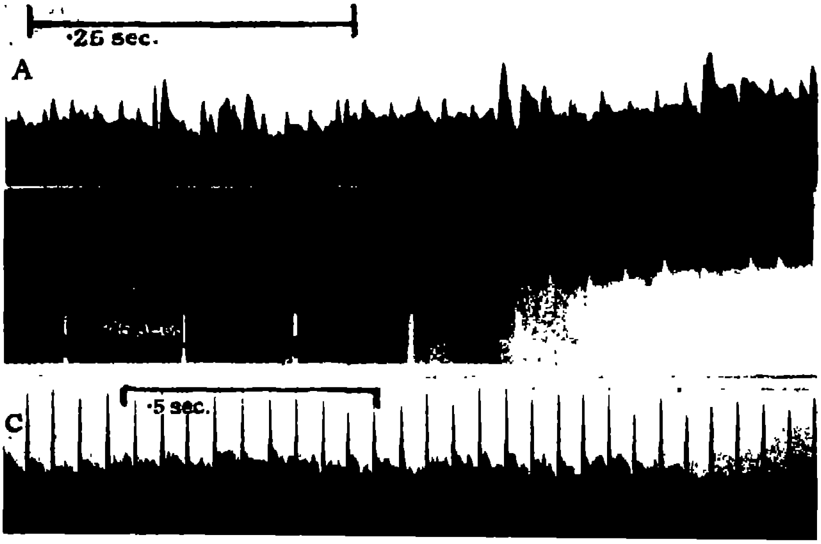

Fig. 1. Typical records of impulse discharges in different kinds of nerve fibre. A, sensory discharge in a mammalian nerve fibre leading from a Pacinian Corpuscle (stimulated by pressure) (Adrian and Umrath (1929)). B, motor discharge in a mammalian nerve fibre concerned in maintaining the contraction of the thigh muscles (Adrian and Bronk (1929)). C, Discharge in nerve cord connecting two of the segmental ganglia in the central nervous system of the caterpillar. Isolated preparation, no external stimulus (Adrian (1930, *b*)).

cells; it can scarcely depend on special structures developed in the sense organs or in the grey matter of the central nervous system, for discharges of the same general character may arise from the cut end of a nerve fibre (Adrian, 1930). It certainly depends on something which can be made to behave with quite machine-like regularity. Matthews (1931) has recently published an extended study of the muscle spindle in the frog and shown that, if sufficient rest periods are allowed, the whole course of the response to a given stimulus can be predicted with an accuracy of about 1 per cent. Such regularity of behaviour is rare enough in any preparation of living cells, but Eccles and Sherrington (1931) have found the same mechanical precision in some of the reactions of the motor neurone. The exact mechanism remains to be worked out. As a corrective to undue optimism it may be recalled that there are other rhythmic activities such as the heart beat and the waving of cilia which are not yet explained, although they were known long before the rhythmic nervous discharge—and when all is said we are still on the outskirts of the central nervous system, for the rapid transmission of impulses is a very humble part of the activity which transforms a collection of cells into a living animal.

Grading of Activity in the Nerve Fibre.

It has been stated that the impulses in a single nerve fibre are all alike whatever the stimulus which has set them up. This is a point of great importance in the study of the sensory apparatus for it implies that the message in any one fibre can only be varied by changes in the frequency and number of the impulses.

If we are entitled to assume that a change in the character of the impulse would produce a corresponding change in the action potential, the evidence is clear. In two cases sensory discharges have been studied in preparations known to contain only one end organ of a given type; Adrian and Umrath (1929) have worked with a single Pacinian corpuscle in the cat and Matthews (1931) with a single muscle spindle in the frog and with both the potential changes are unaffected by the strength of the stimulus though their frequency varies over a wide range. In other preparations it is not possible to ensure that only one end organ is stimulated and in records made without special precaution there are usually several distinct series of action potentials, each with its own frequency and with potentials of fixed size. The natural interpretation is to suppose that each series represents the activity of a different nerve fibre and this is supported by the fact that the action potentials belonging to one sequence may appear so close to those of another sequence that they cannot be travelling in the same nerve fibre (since the interval between them is shorter than the refractory period). In spite of this there was a remote possibility that different end organs attached to one and the same fibre might produce impulses of different form, but this seems to be ruled out by some recent experiments of Cattell and Hoagland. They find that in the frog the tactile endings of each cutaneous nerve fibre are distributed over a wide area but there is no sign of any change in the action potentials when different parts of the area are stimulated.

It is conceivable that the impulses might vary without any corresponding variation in their action potentials, but conceivable only on the assumption that our ideas of nervous conduction are quite incorrect. The hypothesis which holds the field, and seems likely in its main outlines to continue to hold it, makes the potential change an essential link in the transmission of the active state from one section of the nerve fibre to the next. On this "membrane hypothesis" the local currents cause the spread of the surface breakdown into the inactive regions and make these active and in their turn the seat of further electrical change. Thus the activity of each section of the nerve fibre is a direct consequence of the potential change in the preceding section; similar potential changes must produce similar periods of activity and if the potential change does not vary with the stimulus there is no possibility of variation in the impulse. The membrane hypothesis may turn out to be wrong, but there is nothing in the records of nerve discharges to throw any doubt on it and there is no other good evidence to suggest that the action potential and the impulse are not directly related to one another.

It is worth while insisting upon the unvarying character of the nerve impulse as revealed in records of motor and sensory discharges, for there are signs of a reaction against the doctrine usually known as the "All-or-None Principle." The reaction is natural enough and the phrase itself is largely to blame, for a statement that a nerve or a muscle obeys this principle can be given a much wider interpretation than has ever been justified by the facts. The activity of a nerve or a muscle can be graded both by alterations in the number of fibres in action and in the frequency with which the impulses succeed one another; moreover, the size and form of the impulses can be varied by changing the condition of the structure in which they appear, *e.g.*, by alterations of temperature, mechanical deformation, etc. All that can be asserted is the much less sweeping statement that there is an all-or-nothing *relation* between the stimulus and the propagated disturbance which it sets up. The wave of activity which travels away from the stimulated region is one which occupies the entire resources of the fibre at each point; it depends only on the local condition of the fibre and not on the strength of the stimulus. But in the area directly affected by the stimulus other changes will occur, and these may be of graded intensity. Examples are the local change which follows an inadequate stimulus in muscle or nerve and the localised contraction which appears at the cathode in muscle during the passage of a constant current (*cf.* Lucas, 1908). Changes of graded intensity may well occur in the regions from which impulse discharges arise, the sense organs or the central grey matter; their intensity will affect the frequency of the discharge, but it is safe to conclude that it will have no influence on the size of each impulse in the nerve fibres.

Changes in Impulse Frequency: the Touch Receptors.

The simple character of the sensory message raises a problem which in a slightly different form has been under discussion for many years. In any one nerve fibre the discharge may alter in frequency but in no other way. What then will be the effect of a change of frequency on the sensation which the message evokes? There is good reason to suppose that changes in the intensity of sensation depend on changes of frequency in the message, but can the frequency determine the quality of the sensation as well as its intensity? Increasing pressure with a hard object on the skin makes us feel first touch, then pressure and finally pain. Are these sensations produced by discharges of increasing frequency in the same group of sensory fibres, or is each kind of sensation due to a specific nervous apparatus? For the special sense organs this query was answered many years ago by Johannes Müller in his statement of the doctrine of "specific nervous energy." For the sense organs in the skin it has been answered by von Frey, who holds that the skin surface can be mapped into small spots, each with a particular quality of sensation attached to it. However it is stimulated a touch spot will give the feeling of touch and a cold spot that of cold. The pain spots are very widely distributed, but von Frey considers that they produce no other sensation than that of pain. On this point, however, there is still some difference of opinion, for although the distinction of thermal and mechanical receptors is generally admitted, the specific character of the pain receptors is not, and Goldscheider and his school consider that pain may arise from intense stimulation of the receptors for mechanical change (Goldscheider, 1926).

The study of the impulse messages has not yet given much that is conclusive, but pain is so important an element in our lives that the results seem to be worth stating if only to show what may be expected of this line of attack.

The first conclusion is that the receptors for light touch are supplied by the larger medullated fibres and that in some at least of these fibres no messages can give rise to pain in the normal animal. The evidence for this is simple enough. When the frog's skin is touched with a wisp of cotton wool each contact and each movement of the wool along the skin surface produces a short outburst of impulses in the cutaneous nerves. The action potentials are not all of the same size, but they are conducted rapidly and must be due to the larger nerve fibres. Matthews (1929) has found a rate of conduction of 15 metres a second at 15° C., and this probably corresponds to a fibre diameter of 7–10 μ according to the principles of analysis used by Gasser and Erlanger (1927). By a special technique it is possible to record only those impulses which are travelling in a particular nerve fibre. It is then found that the discharge in response to a touch may vary from a single impulse to a dozen or more according to the rate of movement of the skin surface, but the receptors become adapted so rapidly that persistent light contact has no effect after the initial skin movement is over. Under normal conditions, therefore, the messages from the tactile receptors will be intermittent and the frequency will never be maintained at a high value. But it can be maintained at a very high value by the use of a rapidly fluctuating stimulus. Cattell and Hoagland have used an air jet interrupted by a toothed wheel and have found that each puff of air will produce a single impulse. The discharge can be maintained at frequencies of 250–300 a second for a quarter of a second, and at slightly lower frequencies for much longer times. Stimulation producing such a continued high frequency discharge must call on the nerve fibre to the limits of its capacity, for its refractory period will prevent it responding at higher rates. There is no way in which the message could be intensified, yet it is clearly not painful, for an intact frog pays no more attention to an interrupted air jet than it does to a touch.

The same result can be shown with the cutaneous nerves of the guinea pig. The receptors at the roots of the hairs agree with the tactile receptors of the frog in their rapid adaptation, and in the large size of the nerve fibres which supply them. When the hairs are touched with a steel strip vibrating at several hundred times a second there is a continued impulse discharge at the same high frequency. Yet an intact guinea pig certainly does not behave as though it felt pain when stimulated in this way, and we ourselves would feel nothing more than vibration or tickling.

Thus the nerve fibre which conveys the message aroused by light touch cannot be responsible for the pain aroused by severe pressure. To this extent the distinction of touch spots and pain spots (or touch fibres and pain fibres) is fully confirmed.

Nerve Fibres responsible for Pain in the Mammal.

In the mammal there is fairly clear evidence that some of the messages which arouse pain travel in nerve fibres which do not differ greatly from those connected with the tactile receptors, though they are probably smaller and their rate of conduction, though rapid, is not as high as that of the tactile fibres. Erlanger and Gasser (1930) have shown that the sensory fibres can be divided into a group conducting rapidly, group A, and one conducting very slowly, group C. The fibres in group A conduct at rates which vary from 80–30 metres a second in the mammal, and those in group C conduct at 1·3–0·7 metres. Now there are various protective reflexes which are evoked by sensory messages travelling at 30 metres a second or more. Dennig (1929) found a rate of 40 metres for the sensory fibres in the splanchnic which produce reflex contractions of the abdominal muscles, and Eccles and Sherrington (1931) one of 30 metres a second for the sensory fibres concerned in the flexion reflex of the leg. It may be noted incidentally that distension of the bile duct causes a large outburst of impulses in the splanchnic and that these are certainly of the rapid type judging by the form of their action potentials (fig. 2, Plate 2). It is possible that the message which gives rise to a protective reaction is not the same as that giving rise to pain, but it is interesting to find that many of the fibres which conduct at a rate of 30–40 metres a second are particularly liable to give a persistent discharge when they are injured (Adrian, 1930). In fact their activity is so great that it is difficult to work with the larger mammalian nerve trunks because so many of these fibres must be injured in lifting the nerve from its bed. These persistent discharges are likely to cause pain if they cause any sensation, but the fibres in question are mainly distributed to the blood vessels and connective tissue and they are not often brought into play by injury to the skin surface. They are probably of small diameter (4–6 μ) for their action potentials are distinctly less than those due to the muscle receptors or the tactile organs at the roots of the hairs, but their rate of conduction qualifies them for the rapid A group of Erlanger and Gasser.

We meet with impulses of small action potential when the skin surface is stimulated, but here the nature of the fibres is not so clear. The brilliant work of Sir Henry Head has shown that with certain nerves such as the ulnar the area supplied with receptors for protopathic sensation (pain, extremes of temperature, etc.) is much larger than that for epicritic (light touch, etc.);

this arrangement makes it possible to compare the messages of the two kinds of receptors, for stimulation at the margins of the receptive field should take effect on the protopathic receptors alone. The experiment has been carried out on nerves in two situations, the ulnar of the cat and the dorsal cutaneous nerves of the guinea pig, and with both there is a pronounced difference in the response from the centre and margins of the receptive field. A light touch on the ulnar side of the cat's paw gives impulses of large action potential probably conducted at 50 metres a second or more, and in addition to these there are usually impulses of much smaller potential, at any rate when the stimulus is enough to cause some deformation of the skin surface. On the median side of the paw deformation of the skin gives the small impulses but not the large, and there is a sharp line of demarcation between the two parts of the receptive area (fig. 3 and fig. 4, A and B, Plate 2). The same result is found with the guinea pig, for each dorsal cutaneous nerve supplies a central zone giving large and small impulses and a peripheral zone giving only the small (fig. 4, C and D, Plate 2).

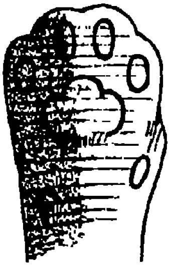

FIG. 3. — Receptive fields on the cat's paw giving action potentials in the ulnar nerve. Stimulation of the dotted region gives large action potentials (*cf.* fig. 4, A) as well as small; stimulation on the median side of the paw gives only the small potentials (fig. 4, B).

The small impulses are certainly conducted less rapidly than the large, and the records suggest that they may travel as slowly as 15–20 metres a second, though accurate measurements have not been made. There can be little doubt that the impulses travel in nerve fibres of small diameter, that these have a wider peripheral distribution than the large fibres and that, with the ulnar, the distribution of large and small fibres agrees with the distribution of epicritic and protopathic sensitivity in man. Whether the small fibres should be classed as protopathic is largely a matter of definition. Their receptors are stimulated by a movement of the skin which would not cause pain in a normal animal, but this is perhaps no reason against their belonging to a system which registers contact as painful under the abnormal conditions which exist when a nerve has been cut through. The receptors giving the small impulses are somewhat less sensitive to movement than those giving the large, and their character and distribution would agree with Goldscheider's notion of a class of receptor which can give pain under intense stimulation. But most of them become very rapidly adapted to a constant stimulus and for this reason the discharge

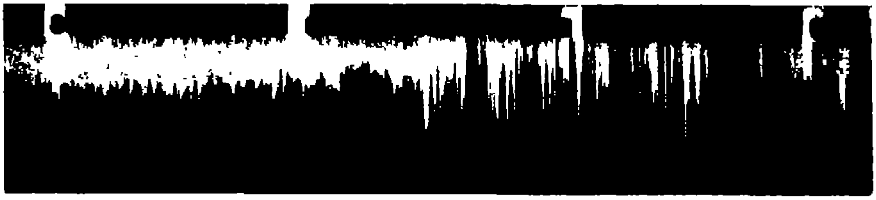

Fig. 2. Nervous discharge in the afferent fibres of the splanchnic nerve (cat—decapitate preparation). The great increase in activity is caused by distending the gall bladder and bile duct with air. The potential waves are due to large fibres which conduct rapidly.

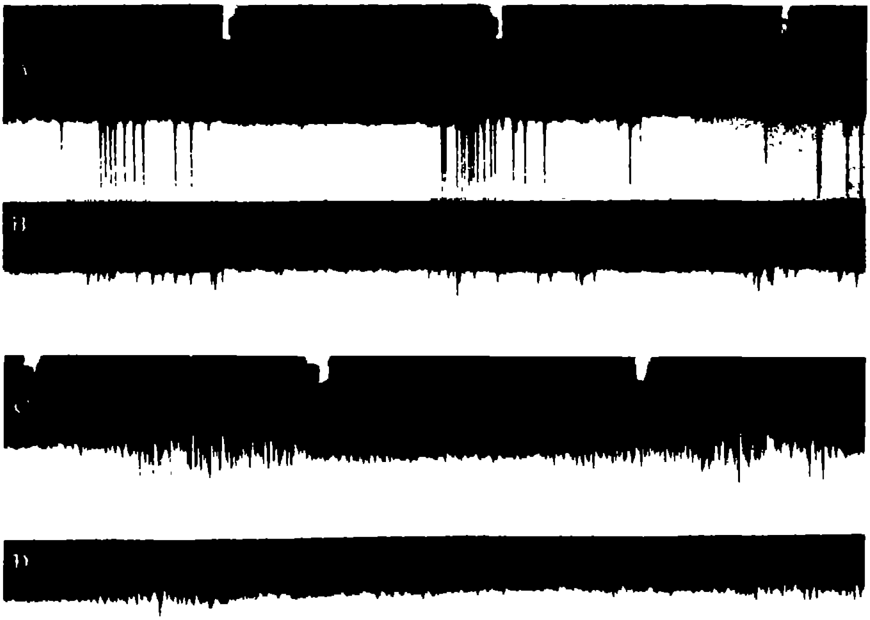

Fig. 4. A and B, discharges in a branch of the ulnar nerve (cat—decerebrate preparation). A, large action potentials due to touching the ulnar side of the paw; B, small action potentials due to touching median side. C and D, discharge in a dorsal cutaneous nerve of the guinea pig. C, large and small potentials due to touching the centre of the receptive field on the skin of the back; D, small potentials from the margin of the receptive field. *The form of the small waves shows that the impulses are* conducted less rapidly than the impulses giving the large potentials. In these and the following records the time marker (white marks at the top of the strip) gives intervals of 0·25 second.

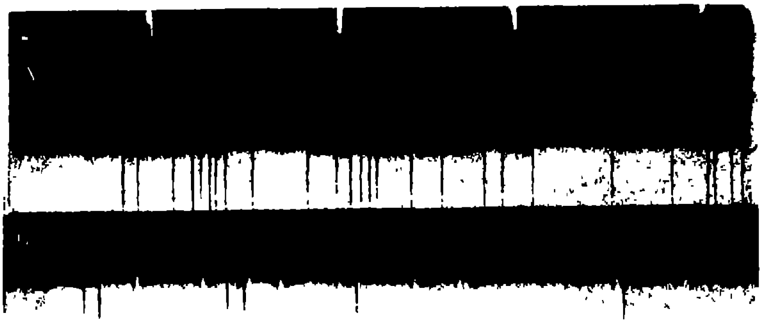

FIG. 5. Discharges in dorsal cutaneous nerves of the frog (*R. temp.*). A, large action potentials due to touch; B, effect of 2 per cent. acetic acid on skin. Persistent discharge of very slow impulses giving a small change of potential.

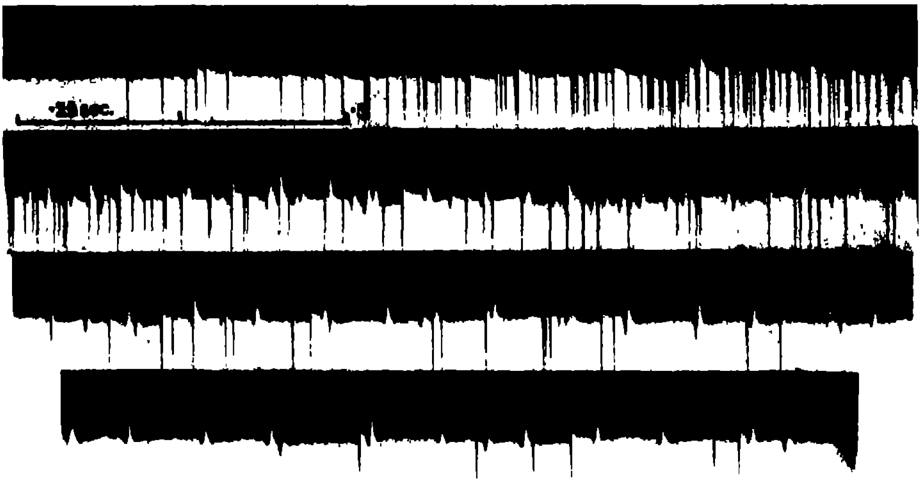

FIG. 6.—Effect of increasing pressure on the frog's skin. A, B and C are consecutive strips and show the appearance of the slow impulses 1 second from the start of the discharge. Record D was made 6 seconds after C and shows the persistent discharge of slow impulses under a steady pressure (of considerable intensity).

produced by cutting, burning or crushing the skin seems scarcely adequate to account for the pain which we should feel from an injury of the same kind. There is an intense discharge of both small and large impulses (if the central zone is affected) during the infliction of the injury but nothing to correspond to the persistent dull pain which would follow. Again, if the skin surface is scraped with a knife and then covered with weak acid there is often no discharge at all, or at most a momentary outburst as the acid runs over the skin.

Thus the receptors which give the small impulses respond to contact and movement as well as to stimuli of damaging intensity and their response to the latter suggests that the essential stimulus is the momentary deformation rather than the damage. They may well be responsible for the acute pain felt at the moment of injury, but something more seems to be needed to complete the picture. Unless the skin of the cat and the guinea pig is much less sensitive than our own, we must conclude that the discharge does not represent all that is taking place in the nerves—that they are transmitting pain messages which we cannot record.

A possible explanation is that the messages may be carried by fibres which give exceedingly small potential changes. Two years ago Erlanger and Gasser (1930) showed that a mixed nerve trunk contains a group of fibres which conduct very slowly and give a very small potential wave. They form the C group in their classification with a conduction rate (in the mammal) of 0·7 to 1·3 metres a second, as against the rate of 30 metres a second and more for the fibres of the A group. Some of the fibres in the C group belong to the sympathetic system, and there is a group (B) conducting at intermediate rates which also belongs to this system, and does not enter the dorsal roots. But many of the C group enter the dorsal roots and are presumably sensory, and as they have a very high threshold to electrical stimulation they might well form part of the afferent mechanism for pain. At present their activity has only been detected in the mammal when the nerve is stimulated electrically so that all the fibres are in action together. We may hope that with improved technique it will be possible to record the activity of individual fibres of this type, but at the moment the sensory discharges have not revealed any potential waves with a rate as slow as 1·3 metres a second. But the search in mammalian nerves has not been exhaustive, for these very slow impulses can be recorded in the nerves of the frog. Here they supply just what is lacking in the mammalian records and it has seemed most profitable to explore the slow system in the frog as thoroughly as possible before returning to the search for it in the mammal.

The Slow Fibre System in the Frog.

The impulses set up by tactile stimulation of the frog's skin have been mentioned already. They travel in the larger medullated fibres at a rate of about 15 metres a second (corresponding to one of about 50 metres a second in the mammal) and their receptors become adapted very rapidly to a constant stimulus. The action potentials are not all of the same size and on the whole the potentials obtained by stimulating in the centre of the receptive area of a nerve are larger than those from the periphery. To this extent the arrangement is comparable to that of the skin receptors in the mammal, though in the frog there is no clear difference in conduction rate, and the division into large and small impulses may depend mainly on the greater branching of the fibres which supply the periphery.

A light touch with cotton wool or a brush gives a discharge which consists entirely of these rapid impulses with relatively large action potentials, and in several earlier investigations (Adrian, 1926; Matthews, 1929) it appeared that a pin prick or a firm pressure gave no more than a prolonged discharge of impulses of the same sort. Occasional slower impulses were noted, but their appearance was capricious. Our failure to detect the action potentials of the very slow system of nerve fibres was probably due to lack of familiarity with them. Although the sensitivity of the amplifying system is no greater, it is now an easy matter to record the slow impulses even in the larger nerve trunks, though the potential changes are much less than those of the rapid fibres and for this reason the best results are given by the slender dorsal cutaneous nerves where the potential changes due to each fibre are relatively large. The slow potential waves are absent with light touch, but are invariably present with stimuli which would damage the skin, and the list of stimuli includes the application of weak acid and of heat as well as various forms of mechanical injury.

A selection of records in which they appear is given in figs. 5–10 (Plates 3–5). In many of these records the amplifier is working at or near its maximum sensitivity and the base line shows a number of small irregular fluctuations which are due to the random fluctuations in the plate current of the first valve (shot effect, etc.). They represent changes of about 1 microvolt, and as they are developed within the amplifier they prevent us from recording tissue changes of the same order of magnitude. Fortunately the slow potential waves are quite large enough to appear distinctly, and in many records they reach 10 microvolts or more. They may be seen without difficulty in fig. 5,

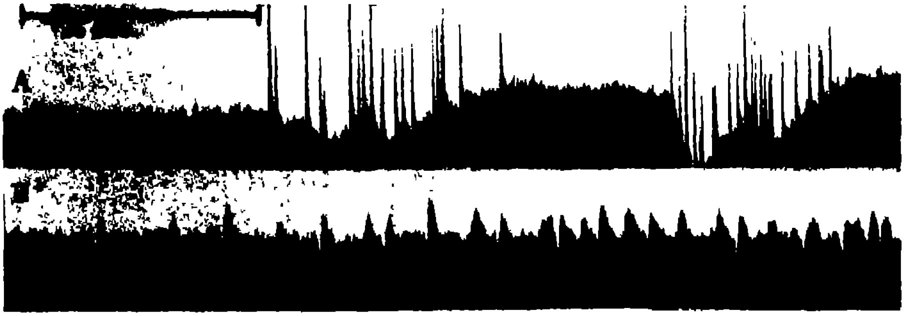

FIG. 7. Dorsal cutaneous nerve of frog. A, large, rapid action potential waves due to touch; B, skin scraped with a knife to remove surface layers and then crushed in a screw clamp. The discharge consists entirely of slow impulses.

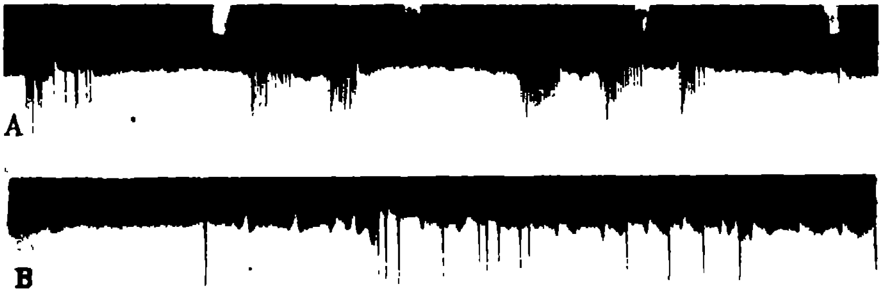

FIG. 8. Another preparation. A, impulses due to touch with cotton wool. B, pin thrust into the skin near the edge of the receptive field.

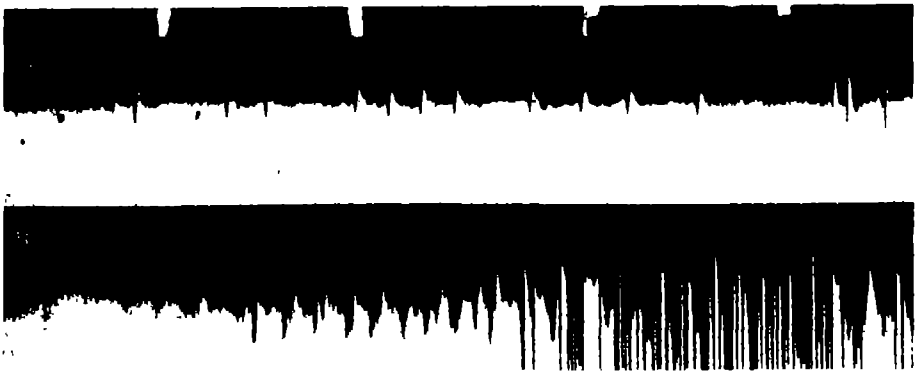

FIG. 9.—Effect of burning by holding a hot soldering iron near the skin (not near enough to cause any visible change). In A the iron was held at some distance; in B (from another frog) it was much nearer. The slow fibres come into play before the rapid in both records.

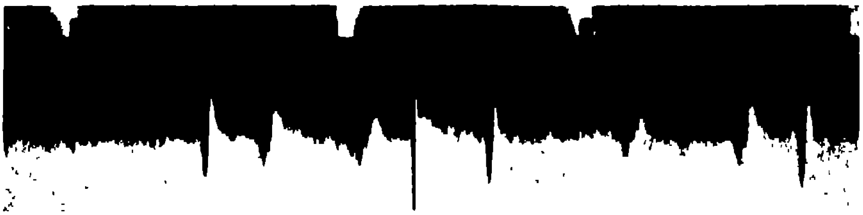

FIG. 10.—Impulses due to pressure at the edge of the receptive skin field showing the variation in the size and form of the waves. The smaller the change the slower it travels. The very small oscillations of the base line are due to the amplifier and not to changes in the nerve.

in which the upper record shows the effect of brushing the skin with cotton wool and the lower the effect of applying 2 per cent. acetic acid on a fragment of filter paper. Owing to the difficulty of obtaining a suitable nerve of greater length than about 1 cm. the potential changes under the two electrodes are not distinctly separated, but some idea of the rate of conduction is given by the interval between the ascending and descending phases of each response. The responses fall into two distinct types, large excursions due to the rapid fibres in which the two phases succeed one another so quickly that the whole response is reduced to a thin vertical line, and small excursions which leave a much wider trace on the record.

The different sensitivity of the slow and fast systems to mechanical stimulation can be seen very clearly in fig. 6 (Plate 3). To make this record the skin was arranged on a rigid surface and pressed with a vulcanite rod moved up by rack and pinion. For the first second the increasing pressure stimulates only the rapid system; the slow system then comes into play and the record shows a confused medley of fast and slow excursions. Under a maintained pressure the discharge of rapid impulses declines after the first second to an occasional irregular firing, but the slow impulses continue without much reduction in frequency. Fig. 7 (Plate 4) show a result of the same kind, but in this case the rapid system had been partly put out of action by scraping the skin surface with a knife. This treatment gives a persistent discharge of slow impulses and causes a temporary failure of the rapid system which allows the activity of the slow fibres to be made out more clearly. In the lower record the scraped area of the skin was compressed in a screw clamp at the moment of recording. The discharge consists entirely of slow impulses—rapid impulses due to touching another part of the receptive field are shown in the upper record for comparison. Fig. 8 (Plate 4) records the response to touch and to a prick with a fine needle, and fig. 9 (Plate 5) shows two records of the effect of holding a hot iron close to the skin. Here it will be seen that the slow fibres come into play before the rapid; indeed, the activity may be confined to the slow system alone if the heat is only applied for a short time.

The distinction between the slow and rapid systems is clear enough, but the slow impulses do not all travel at the same rate. This may be seen from fig. 10 (Plate 5), which gives a group of impulses set up by heavy pressure at the extreme edge of the receptive field where there are relatively few endings belonging to the nerve under examination. The form of the slow waves varies considerably and there is evidently a general parallelism between the rate of conduction and the magnitude of the potential change in the different fibres, for the smallest responses are those with the greatest separation between the two phases. Without a knowledge of the monophasic wave form we cannot make an accurate estimate of the rate of conduction, but other experiments have given values ranging from 0·3 to 2·5 metres a second. These are rough approximations and although a rate of 2·5 metres a second is well above the upper limit of Erlanger and Gasser's slow C class (which conduct at 0·3–0·6 metres a second in the frog) the agreement is close enough if we take into account the great difference in methods and data. The difficulty of measuring the conduction rate accurately is enhanced by the unusual form of the potential waves. These are generally triphasic with the second phase much larger than the first, a form which is probably due to a branching of the nerve fibres on their way to the skin.

It is interesting to find that the skin area supplied by the slow fibres corresponds fairly closely with that supplied by the rapid. There are occasionally small patches at the margin of the receptive field which are supplied by the slow fibres alone, but the boundaries of the two systems usually coincide to within 1 mm. There is, however, a great overlapping in the distribution of the fibres from different nerve trunks in the frog and any point on the skin of the back usually falls within the territory of at least two of the spinal nerves and of many cutaneous branches. The coincidence in the receptive fields for fast and slow fibres is found in the case of the internal saphenous nerve as well as the nerves of the back, though here also the field for the touch fibres overlaps that of other nerves.

The Slow Fibre System in Relation to Pain.

The kind of stimulation needed to excite the slow fibre system shows that it will only come into play in conditions which would give rise to pain, and the persistence of the discharge for several minutes after a severe injury agrees with our experience of pain after injury to the skin. Erlanger and Gasser have shown that many of the slow fibres are sensory in that they enter the cord by way of the dorsal roots. We may fairly conclude, therefore, that the messages in the slow fibre system will produce pain or its physiological accompaniment. It must be admitted that the evidence is indirect and that there may be many pitfalls in an argument which equates sensation in man with nervous discharges from the frog's skin. For instance, it might seem possible that the slow fibres, which are probably non-medullated, form part of an afferent mechanism for sympathetic reflexes and make no appeal to the rest of the nervous system. But this at least can be ruled out, for if weak acid is applied [illegible] a skin area from which the surface layers have been scraped away, the discharge in the nerve is composed entirely of the slow impulses; yet in the brainless frog this treatment gives rise to vigorous protective movements, *i.e.*, to activity of the skeletal muscles and not merely to visceral reflexes. Thus the slow fibre system is undoubtedly concerned with protective reactions in which the whole body may be involved, and it is a reasonable conclusion that it must be concerned with pain as well.

Yet it does not follow that other kinds of fibre may not also give rise to pain, and indeed the very slow rate of conduction in these fibres makes it unlikely that they are the sole mechanism for protective reactions. Jolly (1928) has measured the reaction time for the flexion of the leg in a spinal frog when the foot is stimulated by a hammer tap or a single induction shock. He gives a reaction time of 18 σ at a temperature of 16° C.; the pathway from the foot to the spinal cord is about 8 cm. long and it would take about 80 σ for a slow impulse to travel this distance. It is possible that the stimulation would not be painful or that the pain might not be felt (assuming that the frog feels as we do) until after the reflex response had begun, but a frog would stand little chance of survival if its protective reactions were all dependent on afferent messages which travel so slowly.

Different Fibre Systems concerned with Pain.

Since electric stimulation of mammalian nerves reveals a group of slow fibres which correspond with those in the frog, it may be inferred provisionally that [illegible] system behaves in the same way in both animals, responding only to intense stimulation and giving a persistent discharge after an injury. In the mammal there is no doubt that some pain reactions are caused by fibres belonging to the rapid "A" group and there is some evidence for this in the frog [illegible]. Taking the evidence from the frog and the mammal together we reach the conclusion that more than one kind of nerve fibre must be concerned; that some of them are probably non-medullated and conduct very slowly and [illegible] medullated and conducting rapidly, though none are as large or as [illegible] as the fibres from the sensitive touch receptors.

[illegible] must be admitted that the evidence for the slow system comes mainly from the frog and that for the rapid system mainly from the mammal. In the frog there is nothing to correspond with the persistent activity which we find in the smaller medullated fibres of the mammal after deep injury,* and there is no clear separation of the receptive skin field into central and periphera zones giving impulses of different rate and size. It must be admitted also that the evidence for the rapid system is concerned not so much with pain as with "pain reactions" or more strictly with the protective reflexes. These would belie their name if they had to depend on the slowest afferent nerve fibres in the body, but it is conceivable that the pain is, so to speak, an afterthought depending on an afferent system of its own. Here we may fall back on evidence of an entirely different kind which has the merit of dealing with pain itself. It is the outcome of some ingenious experiments by Piéron (1929), designed to measure the rate of conduction of the pain pathway or pathways in man. His method was to stimulate the skin at different distances from the central nervous system and to determine the moment at which pain appeared. Three different forms of stimulus were used, pin prick, pinching with spring forceps, and immersion in very hot water; they were applied to the hand, the foot and the forehead, and the subject's reaction times were measured in the usual way. Controls were arranged to ensure that the subject would react to the painful and not to the tactile element in the sensation, and the stimuli were adjusted to produce sensations as near alike as possible from the three regions. Considering the great difficulty of the experiment the results are in good agreement. It appears that the message which carries the pain of a pin prick travels at 15–20 metres a second; that for "burning" travels at 4–5 metres, and that for pinching at 10–15 metres. These specific differences may depend in part on conduction in the pathways within the spinal cord. The range of values does not agree with the conduction rate of either the A or the C group of nerve fibres, and although, as Piéron points out, it does agree with that of the B group, there is the difficulty that no fibres of this group have yet been shown to enter the sensory roots of the cord. But there is one result which stands out quite clearly, namely, that in every case the total reaction time is too short for the "pain" message to be carried by the very slow C fibres.

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* During the breeding season persistent discharges sometimes arise from the cut ends of [illegible] the frog.

The argument for different pain fibres would be more satisfactory if we had evidence from man to show that pain is also produced by fibres of the C group. As it is we have only the evidence that they exist in the mammal and that they evoke pain reactions in the frog. But the rate of conduction of 4–5 metres a second which Piéron finds for burning pain is by no means too high to be compared with that of some of the slow impulses in the frog, and on the whole we may safely accept the conclusion that there is no single afferent system either for pain reactions or for pain itself.

This conclusion may seem a poor return for so lengthy an argument, and the only excuse for the argument is that it brings together a number of facts which have a certain interest of their own. The facts are best left without further comment, for though they invite speculation on such well-worn themes as the protopathic-epicritic classification it is clear that their range must be greatly extended before they can be built up into a coherent system. But there is one line of thought which may be permissible if its speculative character be granted. The feeling of pain can rise to such an intensity that it dominates the whole field of consciousness, yet it seems to depend on the smaller afferent fibres, some of them so small that their very existence has been disputed. The nerve fibres from the tactile receptors are large and conduct rapidly, yet the sensation of touch never rises to great intensity, in fact it can scarcely be said to vary in intensity at all. Both touch and pain are evoked by messages made up of brief impulses which do not vary in size; thus the intensity of the effect must depend on a summation of the changes caused by each impulse. Is it not possible, therefore, that the different character of the sensations of touch and pain may depend in part on a difference in the amount of summation which can occur in the two pathways?

It is at least fairly clear that in the pathway which intervenes between the touch receptors and the mind there is very little smoothing out of the discontinuous activity which we find in the peripheral nerves. A vibrating stimulus gives a message which causes a fluctuating and not a steady sensation. The message consists of volleys of impulses discharged synchronously from a number of receptors and if the frequency is not too high we feel each volley as a separate event. There can be little inertia or momentum (to borrow Sherrington's phrase) in any part of the sensory pathway; there can be little convergence either, for we localise a touch and when two points are touched simultaneously we feel two touches, not one of greater intensity. Thus the rapidity and the privacy of each pathway from the tactile receptors to the mind will prevent any summation of the effects due to each impulse and there will be little opportunity for the sensation to rise to great intensity.

A summation of the effects of individual impulses must be supposed to take place whenever a change of impulse frequency is translated into a change of intensity, and the central nervous system offers many examples of both spatial and temporal summation, the one from the convergence of neurones on a common pathway and the other from the persistence of the effect left by each impulse. Convergence will favour the building up of an intense activity, but in a sensory pathway it will do so at the expense of localisation, for the message must lose its individuality when it enters the common path. A slow decline of the effects contributed by each impulse will favour intensity but will prevent very rapid change. We might expect then that poor localisation and a slow rise and decline would characterise the sensation which can rise to the highest intensity and we find that both are well marked features of pain. It is true that the argument cannot be pressed very far. There are many kinds of pain, some of them fairly well localised and capable of fairly rapid fluctuation, and it cannot be maintained that the most intense are those with the poorest localisation and the slowest rise and fall. Indeed there are so many possible and actual differences between one sensory pathway and another and so many ways in which intense activity might be produced that in the end we may find that the capacity for summation is an unimportant factor. It is, nevertheless, a factor which deserves consideration if only for the reason that it should be easily measurable.

But I must recall the early regulations for the Croonian lecture which were framed to discourage vague and unsubstantial theories. I have now broken them and, I fear, to very little purpose. My excuse must be that speculation of this sort may serve to focus our attention on the relation between physical and mental events. Changes in the nervous system and in the mind belong to such different categories that, in the present state of our knowledge, any comparison may be futile. It is only too easy to forget the complexities on both sides, yet in the end the comparison must be attempted if we wish, even as physiologists, to understand the full effects of a discharge of impulses in a sensory nerve.

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The Vascular Responses of the Eye.

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[Plates 6, 7.]

The investigations described in this paper follow from the theory sponsored by one of us, that the aqueous humour is a dialysate of the blood and the vitreous body is a simple hydrophilic gel (1927, *a* and 1930). During the past four years a series of papers has been published defining the conditions governing the maintenance and control of the intra-ocular pressure in the normal eye (1927, *b*, 1929, 1931); the present paper is an attempt to carry these investigations from the physiological to the pathological aspects of the subject. From our physiological work there emerges the hypothesis that pathological variations in the intra-ocular pressure may depend on two mechanisms: the one involving a vascular upset, and the other dependent upon physico-chemical changes in the vitreous body—and the clinical picture presented by glaucoma exhibits much that is confirmatory to this view. It is with the first of these possibilities that we are immediately concerned.

In considering the possible causes of a vascular disturbance in the eye such as could be responsible for a rise (especially an acute rise) in the intra-ocular pressure, it appeared to us that a fruitful speculation might lie in the activity of a substance corresponding to the H-substance which has been demonstrated by Lewis (1927) and his co-workers to have so widespread and potent effects upon the vascular responses of the skin. By experiments on the intact dog and by the perfusion of the surviving head (Colle, Duke-Elder and Duke-Elder, 1931) we have already shown that histamine has an effect on the vessels of the eye similar to that already determined by Burn and Dale (1926) in the limbs; a dilatation of the minute vessels is associated with a constriction of the more central parts of the vascular tree; when the minute vessels have lost their tone the latter effect predominates, but in a normal state of tonicity the injection of histamine into the circulating blood is associated with a macroscopically visible vaso-dilatation of the capillaries of the inner eye, an increase in the permeability of the capillary walls allowing an excess of colloids to appear in the aqueous humour, and with a marked rise in the intra-ocular pressure.

If a substance of this nature were produced pathologically in the eye, we have to hand all the materials necessary to inaugurate an acute rise of tension.

Two methods employed by Lewis (1927), one of which he believes and the the other he supposes to produce the H-substance in the skin, were selected as being most easily applicable in the case of the eye; the method of stroking, and that of arrest of the circulation. In the experiments the intra-ocular pressure was registered by a manometer which combined in itself a compensatory mercury manometer and an optical manometer which has already been described in a previous publication (Colle, Duke-Elder and Duke-Elder, 1931); blood pressure was recorded in the femoral artery, the state of dilatation of the minute vessels on the iris was observed in the focal beam of a Gullstrand slit-lamp; the rate of blood-flow was correlated with records of the temperature in the anterior chamber taken by thermo-junctions and registered galvanometrically; and the occurrence of an increase in the permeability of the capillaries was shown by the appearance of trypan blue upon the surface of the iris and in the anterior chamber after its intra-venous injection. In the normal animal the capillaries of the eye are impermeable to this dye; it is only detected in the anterior chamber when the permeability of the minute vessels has been increased to a very definite degree, when the presence of a small amount in the anterior chamber is readily visible under the optical conditions employed. In some of the experiments cats were used under ether-chloralose anæsthesia, and in others albino rabbits.

Stroking Experiments.

In the stroking experiments, after the animal had been injected intravenously with trypan blue, the cannula of the ocular manometer was inserted into the eye through the limbus (at " 3 o'clock ") in such a way that its point lay over the iris at the opposite side of the anterior chamber (at " 9 o'clock "). After the intra-ocular pressure had been equilibrated in the usual manner (Colle, Duke-Elder and Duke-Elder, 1931) on the mercury manometer and the optical manometer was alone in communication with the eye, the quadrant of the iris from 7 to 11 o'clock was steadily and firmly stroked with the end of the cannula six to twelve times. It was found possible to do this without any great immediate upset of the equilibrium of the intra-ocular pressure, and the experimental records could always be observed as regular events within 1 minute after the cessation of stroking. Such a procedure was followed by no appreciable alteration in the general blood-pressure with the exception that on several occasions a slight transient fall was observed; but in the eye the following changes were consistently involved.

1. A dilatation of the small vessels is noted after a latent period of about 30 seconds extending markedly over the area of the iris which has been stroked, and especially if the stroking has been firm, after a somewhat longer latent period, and occurring to a somewhat less marked extent, over the entire surface of the iris (fig. 1).

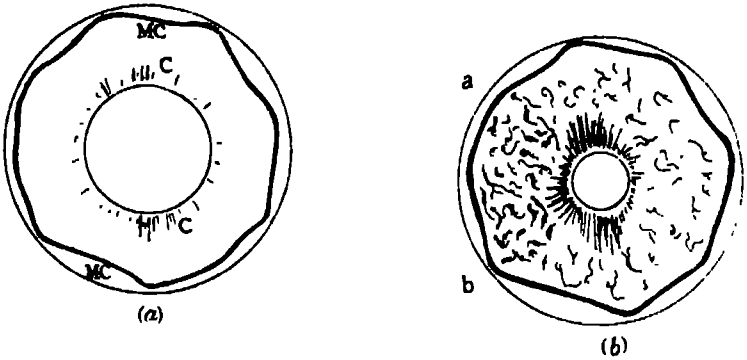

Fig. 1.—(*a*) Diagram of vessels visible on iris of normal albino rabbit. MC, major circle of iris; C, small vessels (arteriolar) round pupillary margin. (*b*) Vessels visible on iris of normal albino rabbit after stroking the quadrant of the iris *ab*.

2. An increased permeability of the minute vessels on the surface of the iris is denoted by the appearance of the violet colour of trypan blue, at first over the area stroked, and then to a less extent over the entire surface of the iris. It appears in 2 to 3 minutes after stimulation, and after 5 minutes the entire anterior chamber takes on a blue tinge, and finally the increase of proteins in the aqueous humour which has escaped from the permeable vessels may be sufficient to form a clot and block the cannula.

3. A rise of temperature is recorded on the galvanometer after a latent period of approximately 60 seconds varying from 0·5° C. to as much as 1·5° C. This falls slowly and gradually, its duration being somewhat variable, and lasting some 10 to 15 minutes.

4. A maximal and constant constriction of the pupil becomes evident some 30 seconds after stroking and persists indefinitely.

5. A rise in the intra-ocular pressure is recorded, starting after a latent period of 2 to 3 minutes. It rises slowly and steadily reaching a maximum of 17 to 30 mm. Hg above the normal pressure after 10 to 15 minutes, after which it tends to fall slightly, never, however, reaching the normal level within the experimental time (fig. 2, Plate 6).

A further series of experiments investigated the extent to which this reaction would repeat itself. If the initial stimulation was small, and the resultant response was feeble, as judged by the transudation of dye and the rise in the intra-ocular pressure, a second more powerful stimulation succeeding the first after an interval of 10 to 30 minutes produced a further rise in the pressure of the eye and a greater transudation of dye. But if the initial stimulus was considerable so that the response attained reasonable dimensions, any repetition of the stimulus, while inducing a further vaso-dilatation of the vessels, was found to be completely ineffective in increasing the transudation of dye and raised the intra-ocular pressure by only a slight amount. This phase of refractiveness was found to persist over the longest experimental periods investigated —over an hour and a half.

A second point of interest which was elicited in experiments subsequent to the occurrence of a well-marked reaction was the action of adrenaline injected into the blood stream. We have already shown that in a normal animal an injection of 0·1 c.c. of 1 in 1000 adrenaline produces a local vaso-constriction in the eye sufficient to present a barrier which prohibits the rise of general blood pressure from becoming effective in raising the intra-ocular pressure. In these circumstances, therefore, we obtained a rise in the general arterial pressure as measured in the contra-lateral carotid and a fall in the intra-ocular pressure. After a well-marked stroking response had been elicited, however, such a fall in the pressure of the eye could never be obtained, but the tracing of the intra-ocular pressure followed passively the changes in the general blood pressure (fig. 3, Plate 6).

A similar series of experiments was conducted after 2 per cent. cocaine solution had been instilled into the conjunctival sac in drops at 2-minute intervals for about half-an-hour. Such a procedure allows sufficient of the drug to permeate into the anterior chamber to effect anaesthetisation. On repeating the stroking in these conditions a very modified response was obtained. Hyperaemia was noted only over the area stroked while the remainder of the iris retained its normal appearance (fig. 4). The increased permeability of the vessels was also confined to this area, and the blue colour was limited to the one quadrant of the iris and never tinted the aqueous humour. The pupil constricted as before, but

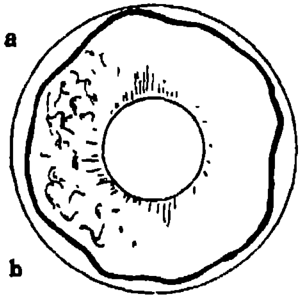

FIG. 4.—Vessels on iris after stroking the quadrant *ab* after cocainisation (albino rabbit).

the rise of intra-ocular pressure was slight in comparison with the previous variation, amounting only to 2 to 3 mm. Hg and falling back to approach the normal level in a few minutes' time (fig. 5, Plate 7).

Apparently, therefore, we are dealing with two types of response, one of which is local in distribution and is independent of nervous activity, and the other of which is general in distribution and takes place only when the nerves are allowed to function. The suggestion at once presents itself that we are dealing with an axon reflex mediated by the trigeminal similar to those described by Bruce (1910) in the conjunctiva. To section this nerve and allow it to degenerate and at the same time to leave the intra-ocular blood supply intact is a procedure involving considerable technical difficulties; but that this axon reflex is a possibility is shown by the effects of sensory stimulation of the cornea. On scratching this tissue with the point of a needle the same reaction results although to a very much less degree than on injuring the iris. The occurrence of this reaction, and its complete absence after anæsthetising the cornea with cocaine, demonstrates the participation of the Vth nerve in the response since branches from this nerve only are found in this tissue.

Occlusion Experiments.

A complete cessation of the ocular circulation is easy to bring about by raising the pressure in the eye to a height above that in the ophthalmic artery. This was carried out and the resultant effects analysed by the apparatus seen in fig. 6. A cannula (A) leading into a glass chamber (B) the distal end of which was closed by a membrane (M) carrying a mirror, formed an optical manometer recording the variations in the intra-ocular pressure by a beam of light from a source (L) on a camera (C). This connected by tubing (D) to a four-way tube, the other three branches of which connected with a horizontal graduated capillary (E), a saline reservoir (F), and a mercury manometer (G) with a mercury reservoir (H). On the distal end of the capillary (E) was a pressure syringe (K) accurately controlled by a screw piston, and a small Hürtle's manometer (M′). The saline reservoir (F) was raised until the anticipated height of the intra-ocular pressure was registered on the manometer (G), and with the saline running from the cannula, it was inserted into the eye. The tap N was then closed and O was opened and the intra-ocular pressure was equilibrated by maintaining an air-bubble in the tube E in a constant position by manipulating the syringe K. After equilibrium had been established and the pressure noted, the tap P was shut off and the normal intra-ocular pressure recorded optically. The tap R was then closed and the reservoir H was raised until the manometer registered a pressure of approximately 200 mm. Hg. With N and O closed, R and P were then opened so that the pressure in the eye was raised to approximately 200 mm. Hg; that it was maintained at approximately this level was verified by a record from the optical manometer. The tap P was then closed retaining this pressure in the eye and the cannula, and the reservoir H was lowered until the manometer recorded the normal intra-ocular pressure. After the pressure in the eye had been maintained at this high level for the required length of time, the tap P was opened, whereupon the chamber of the eye, communicating again with the manometer G, assumed approximately its original (normal) pressure. This was rapidly equilibrated with the air-bubble in E; P was then closed, and subsequent events were observed and recorded optically.

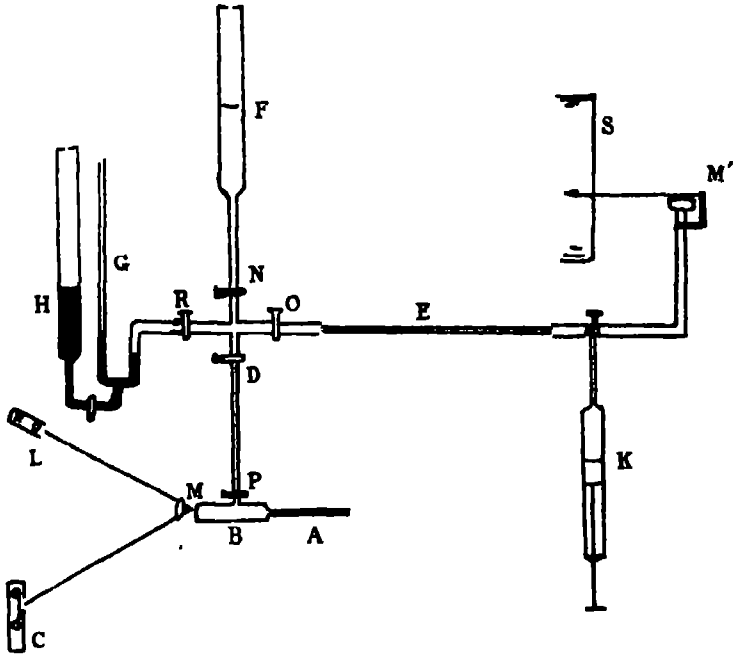

FIG. 6.

In a series of experiments in which superimposed comparative readings were desired, the optical manometer was eliminated and the cannula put into direct communication with the tube E. The same technique was then carried out, but the intra-ocular pressure was recorded by the Hürtle's manometer on the kymograph S.

Occlusion of the circulation brought about a series of phenomena similar in

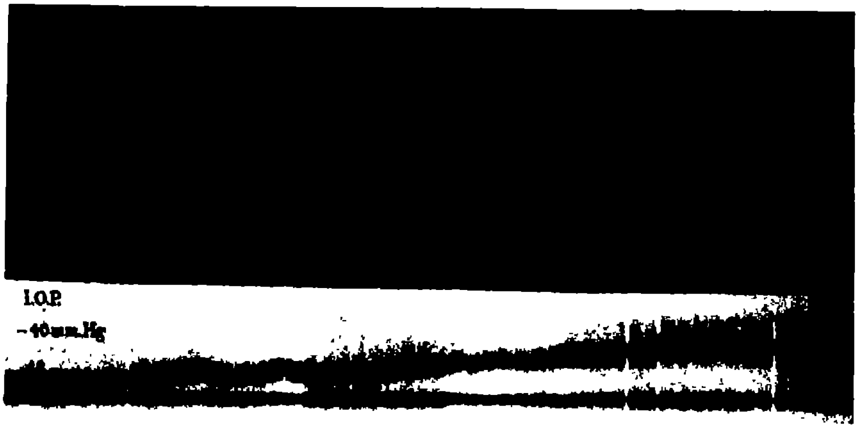

FIG. 2. Reaction to stroking the iris (cat). Upper curve, carotid blood pressure; lower curve, intra-ocular pressure.

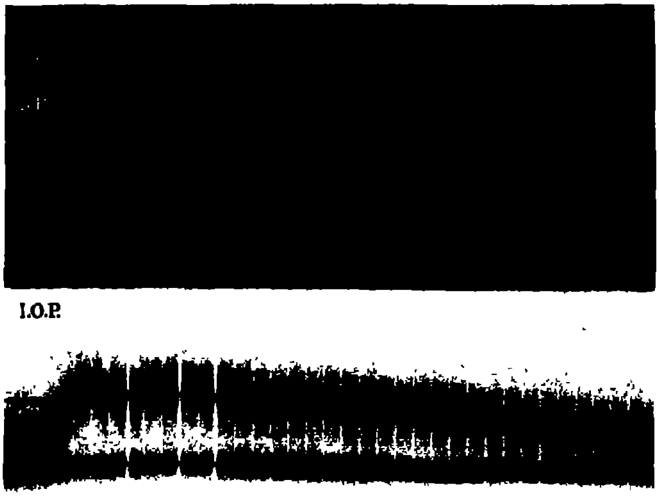

FIG. 3. The effect of adrenaline (0·1 c.c. 1 in 1000) injected intravenously after reaction to stroking (cat). Upper curve, blood pressure in branch of femoral artery. Lower curve, intra-ocular pressure.

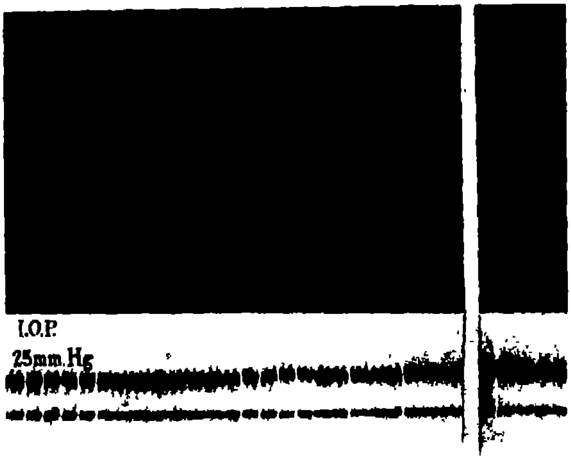

Fig. 5.—The effect of stroking the iris on the intra-ocular pressure after cocainisation (cat). Upper curve, carotid blood pressure. Lower curve, intra-ocular pressure.

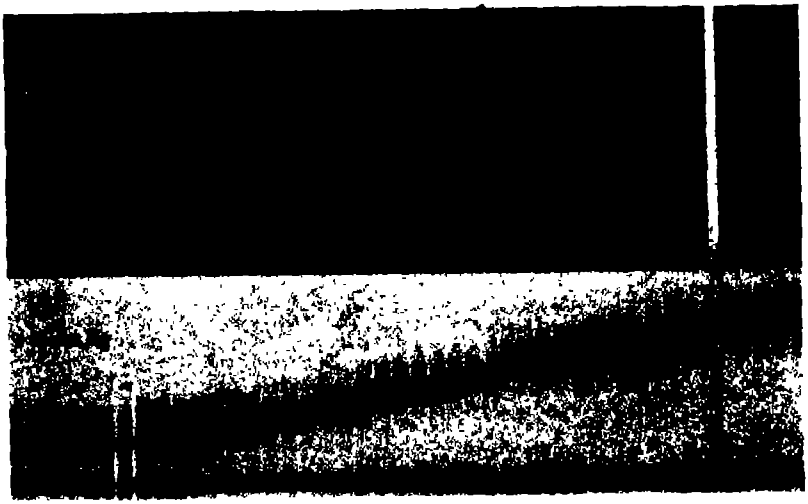

Fig. 7.—Effect of the reactive hyperæmia after occlusion of the ocular circulation (cat). Upper curve, femoral blood pressure. Lower curve, intra-ocular pressure.

nature to those observed with stroking but much more accentuated in degree. Within 30 seconds after the circulation had been allowed to resume, the vessels in the iris were observed to be maximally dilated, the amplitude of the pulse recorded in the tracing became greater, the appearance of trypan blue over the surface of the iris and in the anterior chamber gave evidence of a markedly increased permeability of the capillaries, which after a considerable occlusion was so pronounced that the dye penetrated into the glass chamber of the optical manometer. An increase of blood flow was indicated by a rise of temperature in the anterior chamber of 1° to 2° C., the pupil constricted maximally, and after 1 to 3 minutes the intra-ocular pressure rose sharply as indicated by the optical manometer (fig. 7, Plate 7). The rise of pressure was rapid and for some time after its establishment the amplitude of the pulse was increased; but at a later stage, although the increase in pressure was maintained, the amplitude of the pulse declined. In those experiments in which the initial occlusion had been maintained for periods over 60 seconds (1 to 4 minutes) the intra-ocular pressure tended to remain high or even to increase indefinitely. Again in such experiments the same phenomena of refractiveness and unresponsiveness were evident, for any repetition after a fully developed reaction which involved a marked alteration in the capillary permeability produced no marked rise in the pressure of the eye, while no vaso-constriction followed the injection of adrenaline. The significant difference was noted, however, that occlusion of the circulation after the thorough instillation of cocaine was followed by the same train of events modified not in kind but less marked only in degree.

Discussion.

It is impossible to examine these experimental results without comparing them with the triple response obtained by Lewis (1927) in similar circumstances in the skin. With only minor modifications the two series of phenomena run along parallel lines, and these modifications depend directly upon local conditions. In assessing the meaning of the records of the intra-ocular pressure, it is to be borne in mind that a local capillary dilatation will have little effect, for any increase in the volume of the contents of the globe occasioned by the capillary dilatation will be immediately compensated by the expression of intra-ocular fluid through the safety-valve of the canal of Schlemm (Duke-Elder, 1926). It is important to remember, however, that this applies only to the normal eye; in an eye predisposed to glaucoma wherein the exit channels are inefficient, such an increase of volume would necessarily be reflected in an increase of intra-ocular pressure. A dilatation of the arterioles, on the other hand, is made manifest by a considerable rise in the pressure of the eye since this raises the level of the blood pressure in the capillaries, while an exudation of plasmoid aqueous into the anterior chamber has a similar, although slower, effect by raising the colloid osmotic pressure of the aqueous humour to correspond more closely with that of the blood; such an exudation raises the equilibrium pressure level which is fixed by the difference between the osmotic pressure of the plasma and the aqueous humour on the one hand, and the capillary blood pressure on the other (Duke-Elder, 1927, *a*, 1929, 1931). It is noteworthy that such a rise will be permanent (within the limits of the experimental time) and that so long as an abnormal quantity of colloid material remains in the chambers of the eye, the intra-ocular pressure will remain abnormally high, even although the vaso-motor reaction has subsided. In the first series of experiments wherein the stroke was employed as a stimulus, the local dilatation of the minute vessels of the iris corresponds to the local red reaction on the skin; the increased permeability of the vessels with the formation of a protein-rich plasmoid aqueous humour corresponds to the wheal; and the general dilatation of the vessels obtained on firm stroking, which is abolished when the sensory nerves have been thrown out of action with cocaine, corresponds to the spreading dilatation of the stronger arterioles which is mediated by antidromic nervous activity and which Lewis calls the flare.

In a similar manner the analogy between the effects of circulatory arrest as seen in the eye and in a limb (Lewis, 1927) is very close. There is a dilatation of the vessels with an increased permeability, the occurrence of which is independent of nervous activity, but which is more pronounced when the nerve supply is functionally unimpaired. On the whole the eye of the cat appears to be more sensitive than the human skin. In the latter Lewis and Grant found that while a circulatory arrest of 5 seconds produced a just perceptible volume response in the limb, after an arrest of 1 minute the visible reddening of the skin was still faint, and it required an arrest of 10 to 15 minutes before the response was maximal. In the eye, however, the response after 30 seconds' occlusion of the arteries is very marked, and is maximal after a duration of 1 minute, after which interval "whealing" is very apparent. In both cases also the phenomena of unresponsiveness and refractiveness are evident, whereby in the first case the contractile mechanism appears to be thrown out so that the minute vessels do not contract under the influence of adrenaline, while in the second case no increase in permeability can be obtained after the phenomenon of "whealing" has been well established. In these latter experiments a repetition of the stimulus brought about only a small rise in the tension of the eye such as could be due to an increased vaso-dilatation without the intervention of increased permeability.

In his work upon this subject as shown by the vessels of the skin, it will be remembered that Lewis (1927) deduced that the triple response was due to the liberation of an H-substance having the physiological properties of histamine which was already normally present in the tissues. The occurrence of a slight fall in the arterial blood pressure, the constant development of extreme miosis in every experiment, and the close parallelism of the results obtained are suggestive evidence that some histamine-like substance is concerned in the present reactions. A very interesting point is the demonstration that the complete triple response may occur in an internal organ. While the local red reaction can be elicited on the surface of such internal organs as the liver and spleen in animals (Ebbecke, 1917; Dale and Richards, 1918; Lewis, 1927) or the brain (Florey, 1925), Ebbecke states that the flare is absent from the surface reactions of the viscera and has claimed it to be peculiar to superficial structures such as the skin and conjunctiva. It would appear, therefore, that not only is the eye exceptional in this respect but that this response is even more easily elicited and more marked than in the skin, a peculiarity which can be explained by the occurrence in this organ of axon reflexes which govern the behaviour of its vascular elements. It is well known that the entire uveal tract is plentifully supplied by a nervous network richly interspersed with ganglion cells, and it is upon this basis that the present responses presumably rest. A clinical corroboration of the activity of such a mechanism is perhaps to be found in the profound effects produced by herpes ophthalmicus not only upon the superficial tissues of the eye but upon the uveal tract; elsewhere in the body herpetic lesions occur only in epidermal tissues, and it would seem probable that the severe and intractable iritis which accompanies the disease is directly dependent upon the influence of the trigeminal nerve exerted antidromically upon the intra-ocular tissues. Finally, the acute vascular crises associated with an increased permeability of the intra-ocular vessels and with the destructive rise of pressure which characterises acute glaucoma finds a possible explanation in the consequences of the liberation of an H-substance.

Conclusions.

Injury of the iris by stroking or the suspension of the intra-ocular circulation produces a typical triple response similar in its main characteristics to that occurring in the skin or conjunctiva. Such a response appears to be absent in other internal organs. It is composed of :—

(1) A local dilatation of the minute vessels ;
(2) An increased permeability of the capillary walls ;
(3) A widespread dilatation of the arterioles of the uveal tract.

The first two of these reactions are independent of the activity of the sensory nerves and appear to be due to the local effects of the liberation of an H-substance in the tissues ; the last is directly dependent upon the adequate functioning of the fifth nerve and appears to be dependent upon an axon reflex.

These responses produce a rise in the intra-ocular pressure and may form a factor in the aetiology of certain cases of glaucoma ; they may also form a basis for some of the manifestations of herpes in the inner eye.

We have much pleasure in expressing our indebtedness to Professor Lovatt Evans and Dr. H. H. Dale for their advice in these researches ; and to the Medical Research Council for defraying the necessary expenses by a grant.

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The Absorption Spectrum of the Component c of Cytochrome.

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(From the Biochemical Laboratory, and the Molteno Institute, Cambridge.)

(Received June 25, 1931.)

The component *c* of cytochrome, unlike the other intracellular hæmatin compounds, can readily be extracted and obtained in solution in a form suitable for spectroscopic examination (Keilin, 1930). No quantitative measurements on the extracted pigment have yet been published. Euler (1927) has carried out measurements on a number of yeast extracts containing cytochrome *c*, but these contained other hæmatin compounds in addition. The present paper describes the results obtained with the spectroscopically pure pigment extracted from yeast.

The solutions of cytochrome *c* used in this work were prepared from Delft bakers' yeast by the method previously described (Keilin, 1930), which yields solutions free from other hæmatin compounds. The protein was removed as far as possible by precipitation with sulphur dioxide while the cytochrome was still in the reduced form, and the cytochrome was finally precipitated in the oxidised form with the last fraction of protein. This precipitate, after thorough washing with water, was dissolved in the minimum quantity of alkali and suitably diluted. The resulting solutions had a p_H of between 7·2 and 7·6. In these solutions the cytochrome was mainly in the oxidised form, but a small amount of reduced cytochrome was present. The cytochrome concentration was estimated by reduction and comparison with standard tubes containing pyridine mesohæmochromogen, and the values agreed well with those obtained by estimation of iron content (see Hill and Keilin, 1930). It was estimated that the content of hæmatin (present as cytochrome) was about 1·3 per cent. of the total solids present, the remainder being mainly protein. It is important to note that the cytochrome solutions were non-autoxidisable and did not combine with carbon monoxide, *i.e.*, they consisted of true unmodified component *c* of cytochrome.

In order to obtain the ultra-violet absorption spectrum of the reduced form a small amount of a neutral solution of cysteine was added to the solutions. Sodium hydrosulphite cannot be used as the reducing agent, on account of its very strong absorption in the ultra-violet region. Cysteine is free from this objection. A short time after the addition of cysteine the cytochrome was completely reduced, as shown by comparison with a sample of the same solution reduced with hydrosulphite. The curve shown in fig. 1 was obtained with such a solution.

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* Beit Memorial Research Fellow.

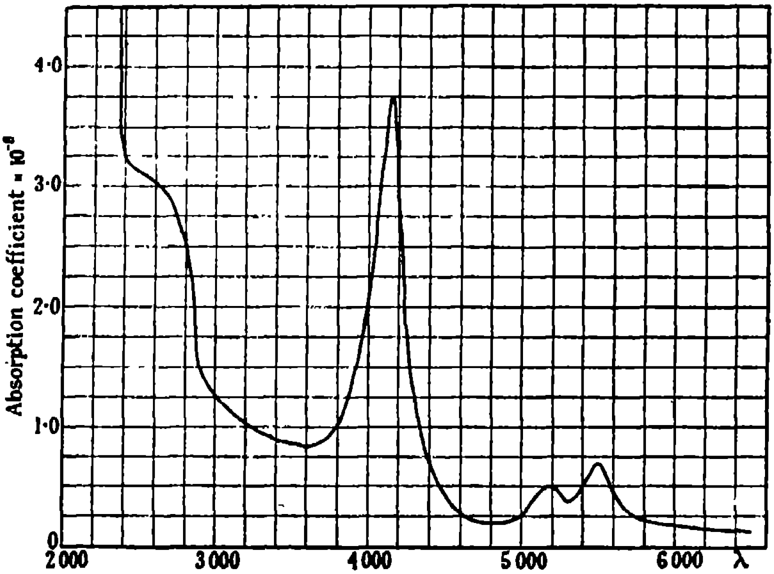

Fig. 1.—Absorption spectrum of reduced cytochrome c. Ordinate—absorption coefficient per gram atom of cytochrome iron. Abscissa—wave-length in Ångström units.

For the absorption spectrum in the visible region there is no objection to the use of hydrosulphite, and it was accordingly used to obtain the curve shown in fig. 3.

As already stated, the initial cytochrome solutions were almost entirely in the oxidised form, but it was necessary to use a solution free from the reduced form in order to obtain the spectrum of the oxidised form. For this purpose an amount of alkali was added to the solution just sufficient to make the cytochrome autoxidisable (Keilin, 1930) and on shaking with air a solution was immediately obtained which showed no trace of the α-band of the reduced form. On reneutralisation, the cytochrome once more became non-autoxidisable and incapable of combining with carbon monoxide, showing that it had not been irreversibly modified by the action of the alkali. Such solutions were used to obtain the curves shown in figs. 2 and 4.

The ultra-violet absorption spectra (figs. 1 and 2) were obtained with a Hilger quartz spectrograph with a rotating sector photometer, using a nickel spark as the source of light. The control tube contained water only. The wave-length scale was accurately adjusted by lines of known wave-length throughout the nickel spectrum.

The spectrum in the visible region was investigated in more detail using a Hilger Nutting spectrophotometer, which was likewise carefully calibrated. Figs. 2 and 4 were obtained by this means.

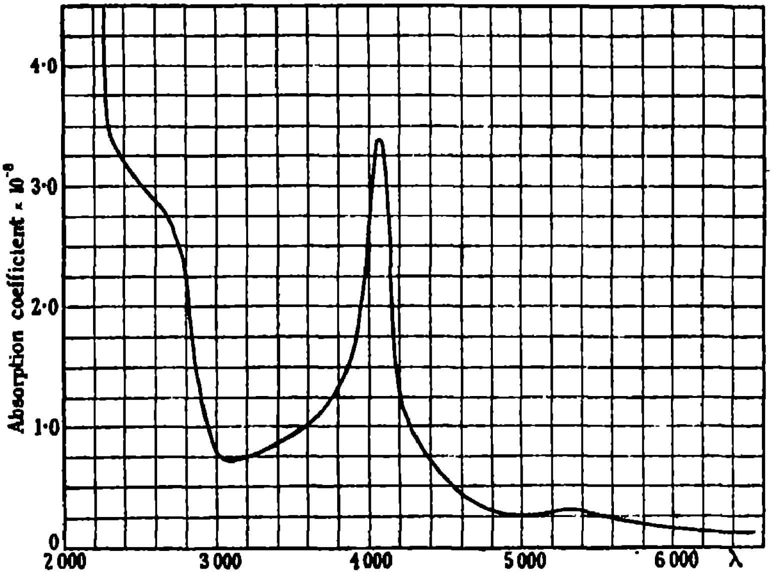

FIG. 2.—Absorption spectrum of oxidised cytochrome *c*. Ordinate—absorption coefficient per gram atom of cytochrome iron. Abscissa—wave-length in Ångström units.

In all the figures (except fig. 5) the "absorption coefficient" is defined as

$$\frac{1}{cd} \ln \frac{I_0}{I},$$

where c = concentration of cytochrome in gram-atoms of iron per cubic centimetre, d = length of tube in centimetre and I_0 and I are the intensities of the incident and transmitted light respectively.

The main feature in the cytochrome spectrum is the intense absorption band in the extreme violet, characteristic of hæmatin compounds. The intensity of this band agrees closely with values obtained by other workers for other hæmatin compounds (see *e.g.*, Hicks and Holden, 1929; Warburg, Negelein and Christian, 1929), and seems to be approximately the same for the reduced and oxidised forms of cytochrome. The position of this band is slightly different in the oxidised and reduced forms, reduction causing a slight but definite shift towards the red end of the spectrum. The maximum absorption

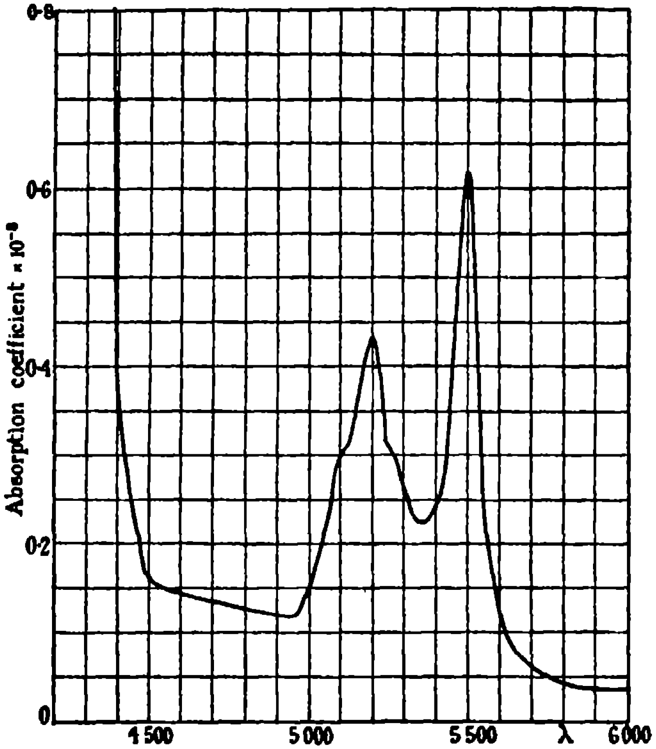

FIG. 3.—Absorption spectrum of reduced cytochrome *c* in the visible region. Ordinate—absorption coefficient per gram atom of cytochrome iron. Abscissa—wave-length in Ångström units.

occurs in the reduced form at approximately 4150 Å., and in the oxidised form at approximately 4075 Å.

Both this band and the α-band of the reduced form at 5500 Å. are simple bands; the β-band of the reduced form at 5200 Å. is however, composite, as shown in the curve of fig. 3. This can also be clearly seen with a direct-vision spectroscope.

On oxidation, the sharp α- and β-bands of the reduced form disappear, and are replaced by a much more diffuse band in the region of 5300 Å., as shown in fig. 4. In a direct-vision spectroscope of small dispersion a weak band is also visible at about 5630 Å., the appearance resembling the spectrum of parahæmatin compounds. It seems, however, that this band is too feeble to show up on the spectrophotometric curve.

It will be noticed in figs. 1 and 2 that there are indications of a further band in the ultra-violet at about 2600 Å. This, however, like the general absorption beyond 2800 Å., is probably not due to cytochrome itself, but to the other substances unavoidably present in the preparations. In order to see whether it was due to the yeast protein, which is the chief impurity in the solutions, we determined the absorption of the protein which was obtained in the purification of cytochrome by precipitation with sulphur dioxide. This precipitate after washing was dissolved by the addition of alkali in the same way as the cytochrome-containing precipitate. It was surprisingly free from cytochrome; even in strong solution no trace of the intense band at 4100 Å. could be detected in any of the plates. The results obtained are shown in fig. 5. It will be seen that there is a marked absorption band at about 2600 Å., in the same position therefore as that indicated in figs. 1 and 2. This appeared to show that the latter band was due to the protein contained in the cytochrome preparations. It seems, however, that it is not due to the protein itself; for if one compares a solution of the cytochrome preparation with a solution containing the same amount of the cytochrome-free protein one finds

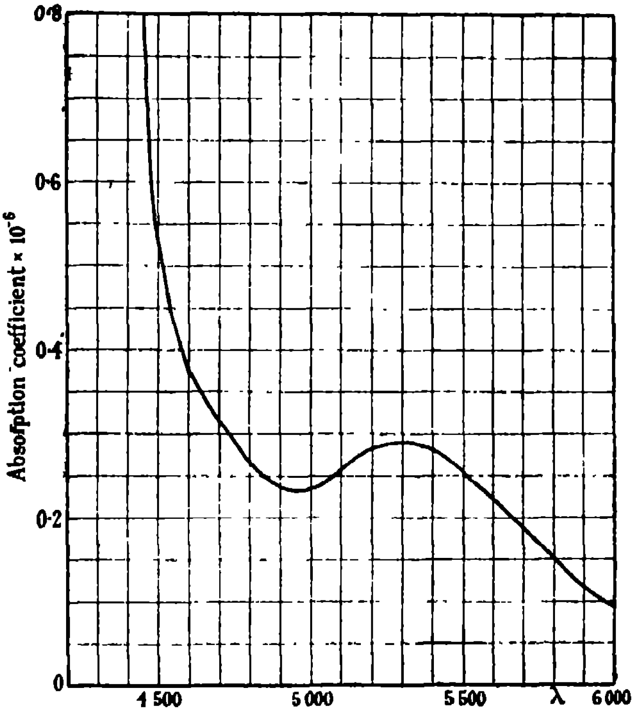

Fig. 4.—Absorption spectrum of oxidised cytochrome *c* in the visible region. Ordinate—absorption coefficient per gram atom of cytochrome iron. Abscissa—wave-length in Ångström units.

that the band at 2600 Å. in the latter case is about 10 times more intense than in the cytochrome preparation. This will be clear from a comparison of the data of figs. 1 and 5, for the cytochrome preparations contained $1 \cdot 86 \times 10^{-6}$ g. atoms of cytochrome iron per gram of protein. It follows that the substance which is responsible for the band is present in much greater amount in the protein fractions first precipitated than in those precipitated with cytochrome. That yeast contains a non-protein substance giving this band is shown by the fact that we obtained in conjunction with Dr. Guha a curve almost identical with that of fig. 5 with a protein-free preparation obtained from yeast (see Guha, 1931)

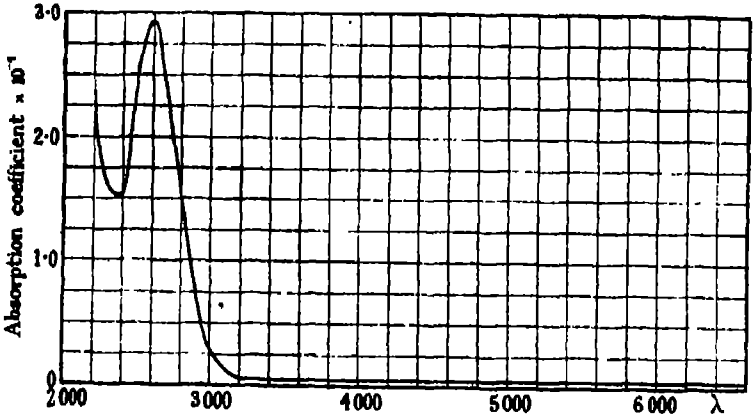

FIG. 5.—Absorption spectrum of cytochrome-free protein fraction. Ordinate—absorption coefficient per gram of protein. Abscissa—wave-length in Ångström units.

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The Oxidation of Hæmoglobin to Methæmoglobin by Oxygen.

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It has long been known that methæmoglobin is slowly formed in solutions of hæmoglobin containing dissolved oxygen but the rate of oxidation has not been measured. With the recent analytical methods of van Slyke and of Conant it is possible to determine whether the decrease in concentration of hæmoglobin is due wholly to the formation of methæmoglobin or partly, as has been suggested, to other reactions, *e.g.*, denaturation and inactivation. The present paper describes measurements of the rate of oxidation at a constant pressure of oxygen using laked blood diluted in phosphate buffers.

Material and Methods.

Blood.—Fresh ox-blood was defibrinated by shaking with glass beads and laked by repeated freezing and thawing. This stock solution was kept at —20° C. and thawed when required. Centrifuging did not alter the concentration of pigment nor did removal of cell fragments by this method change the subsequent rate of oxidation.

Buffer Mixtures.—The K_2HPO_4-KH_2PO_4 buffer mixtures of Cohn (1927) were used. Potassium di-hydrogen phosphate (A.R.) was recrystallised three times, the solution of KOH (A.R.) contained a negligible amount of carbonate. The mixtures were prepared from stock solutions of 1·5 M. KH_2PO_4 and 4·16 M. KOH, the necessary amounts* were taken to give the desired ratio $[HPO_4^{=}]/[H_2PO_4^{-}]$ and the desired concentration of total phosphate after dilution to a given volume with water and a measured volume of laked blood. The p_H of these mixtures at 25° C. and 15° C. was obtained from Cohn's data at 18° C. assuming a temperature correction of $-0\cdot03$ p_H for an increase of 10° C. The mixtures were heavily buffered, the usual concentration of total phosphate was 0·2 M.

The phosphate solution, water and blood, were brought to the desired temperature before mixing. The mixture was placed in a shallow glass vessel moving slowly to and fro (50 times per minute, amplitude 5 cm.) and a constant concentration of dissolved oxygen maintained by passing a slow current of air over the solution. The gas was first passed through wash-bottles containing a solution of similar phosphate composition to that in the reaction vessel. Increase in the rate of shaking or the rate of air-passage did not change the rate of oxidation. Coating the inside of the reaction vessel with paraffin wax had no influence on the rate.

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* Calculated from the equation $KH_2PO_4 + KOH \rightarrow K_2HPO_4 + H_2O$.

Toluene was used as a preservative. There is evidence (*v.i.*) that toluene does not alter the rate of oxidation unless liquid toluene is present as such in considerable amounts. The phosphate solution and water were saturated with toluene and after mixing with the blood one drop of toluene per 50 c.c. of mixture was added. Saturation with toluene was maintained by passing the current of moist air through two wash-bottles containing toluene.

The whole apparatus was contained in a constant temperature room (± 0·015° C.). Portions of the blood solution were withdrawn at intervals and analysed immediately or rapidly frozen at − 20° C. and analysed later. The initial concentration of pigments was determined by analysing a duplicate mixture (p_H 7·65) prepared by adding the blood to water and phosphate solution at 0° C. at the beginning of the experiment. The composition of the mixture, kept at 0° C., did not alter during the time required for analysis.

Methods of Analysis.—The "constant-volume" apparatus of van Slyke and Neill (1924) was used. The graduations on the gas-chamber were calibrated by weighing the mercury contained between them. As the blood solutions were rather dilute the 0·5 c.c. graduation was usually used in the analysis. When the concentration of pigment was small 5·0 c.c. blood solution were analysed, in other cases 2·0 c.c.

The determination of methæmoglobin in the presence of other hæmoglobin derivatives, *e.g.*, denatured hæmoglobin (cathæmoglobin) and hæmatin, depends on the different properties of the reduction products. Methæmoglobin and cathæmoglobin do not combine with O_2 or CO. Methæmoglobin on reduction gives hæmoglobin which combines reversibly with O_2 and CO, cathæmoglobin gives hæmochromogen which combines reversibly with CO but not with O_2. Hæmatin behaves in the same way as cathæmoglobin. Three values therefore may be obtained as follows :—

(*a*) Hæmoglobin—the CO-combining capacity by the method of van Slyke and Hiller (1928).

(*b*) Total pigment—the CO-combining capacity after reduction with sodium hydrosulphite by the method of van Slyke and Hiller (1929).

(*c*) Undenatured pigment—the O_2-combining capacity after reduction with titanous tartrate by the method of Conant, Scott and Douglass (1928).

The concentration of unchanged hæmoglobin is given by (*a*), the methæmoglobin concentration by (*c*)–(*a*), and the concentration of cathæmoglobin and hæmatin by (*b*)–(*c*).

It was found that within the limits of experimental error the values of total and undenatured pigment did not change during the course of the reaction. Under the present conditions therefore the oxidation of hæmoglobin to methæmoglobin is the only detectable reaction. If inactive hæmoglobin (*cf.* Forbes and Roughton, 1931) and denatured hæmoglobin are formed it is only to a slight extent.

Results are given in volumes per cent., *i.e.*, cubic centimetres CO or O_2 (0° C., 760 mm. Hg) combined with or liberated from the pigment contained in 100 c.c. solution. It is necessary to apply a correction (van Slyke and Neill, 1924) which includes the physically dissolved CO or O_2 in the solutions. The correction is determined by repeating the analysis but substituting for the blood solution an equal volume of a solution of the same phosphate composition.* It is assumed that the solubility of CO or O_2 is the same in this solution as in the blood solution and that the corrected value gives the volume of gas combined with the pigment. The correction is large, especially in the case of total and of undenatured pigment but it can be determined accurately.

The agreement between duplicate analyses was within (*a*) 0·07 vols. per cent., (*b*) 0·10 vols. per cent., (*c*) 0·13 vols. per cent. In the case of (*c*) care was required to obtain this agreement; it was essential that the time taken for each step should be the same in different determinations.

An analysis of fresh blood is given below Fresh, defibrinated blood was laked with water, diluted in a buffer mixture at 0° C. and kept at this temperature during analysis.

25 c.c. blood in 100 c.c. mixture
0·2 M. total phosphate
p_H (18° C.) 7·65.

(*a*) Hæmoglobin, 4·45, 4·41; mean 4·43 vols. per cent.
(*b*) Total pigment, 4·39, 4·46; mean 4·43 vols. per cent.
(*c*) Undenatured pigment, 4·45, 4·46; mean 4·46 vols. per cent.

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* To determine the correction for analysis (*c*) the addition of titanous tartrate to the phosphate solution, equilibration with oxygen and analysis were repeated.

The usual agreement between total and undenatured pigment was within 0·1 vols. per cent.

The Rate of Oxidation at 25° C.

Table I gives the results of two experiments at 25° C. with the same stock solution of blood. In this and other experiments the determinations were done in duplicate, the mean values are given below. Times were reckoned from the time of mixing buffer and blood to the mid-time between two analyses. As the reaction was slow the time taken (5 minutes) to transfer the solution to the analysis chamber was negligible; during the time between duplicate analyses (about 35 minutes) the change in concentration of hæmoglobin was very small or negligible.

Table I.—100 c.c. mixture contains 20 c.c. blood, 0·2 M. total phosphate, 25° C.

Experiment 1.

p_H.	Time.	Hæmoglobin.	$\log_{10}(a-x)$.
	hours	vols. per cent.	
5·70	0	3·27	2·000
	15½	1·74	1·726
	24	1·18	1·557
	39	0·52	1·202
6·29	0	3·27	2·000
	16½	2·34	1·855
	40½	1·53	1·670
	63	0·93	1·454
	87	0·51	1·193
6·67	0	3·27	2·000
	18½	2·59	1·899
	41½	2·04	1·795
	64	1·49	1·659
	88	1·14	1·542
	136	0·62	1·278
7·04	0	3·27	2·000
	21	2·76	1·926
	43	2·31	1·849
	65	2·12	1·812
	89	1·81	1·743
	137	1·14	1·542

p_H.	Time.	Total pigment.	Undenatured pigment.
	hours	vols. per cent.	vols. per cent.
	0	3·83	3·79
5·70	46	3·83	3·85
6·29	94	3·75	3·85
6·67	142	3·89	3·90
7·04	142	3·80	3·86

Table I—(continued).

Experiment 2.

p_H.	Time.	Hæmoglobin.	$\log_{10}(a - x)$.
	hours	vols. per cent.	
5·70	0	3·15	2·000
	12	1·92	1·785
	35½	0·67	1·328
6·29	0	3·15	2·000
	13½	2·45	1·891
	37	1·53	1·686
	59½	0·89	1·451
6·67	0	3·15	2·000
	14½	2·65	1·925
	61	1·56	1·695
7·04	0	3·15	2·000
	17	2·78	1·946
	62	1·90	1·781
	90	1·47	1·669

p_H.	Time.	Total pigment.	Undenatured pigment.
	hours	vols. per cent.	vols. per cent.
	0	3·83	3·79
5·70	43	3·86	3·80
6·29	92	—	3·75
6·67	85	3·89	3·86
7·04	109	—	3·90

The concentration of methæmoglobin in the stock solution of laked blood had increased slightly in the interval between the two experiments, probably during the various thawings, etc.

There is no trend in the values of total and undenatured pigment during the reaction at each p_H. The rate of oxidation is therefore given by the change in concentration of hæmoglobin. The last column of Table I gives the logarithm of the percentage of unchanged hæmoglobin. These values are plotted against time in fig. 1, there is good agreement between the two experiments. The linear relation between $\log_{10}(a - x)$ and t suggests that the reaction at a constant pressure of oxygen is monomolecular with respect to the hæmoglobin concentration. The deviation of several of the points is probably due to experimental error, except perhaps at p_H 5·7.

The rate of oxidation was measured twice at p_H 6·29 varying the initial concentration of hæmoglobin over a fourfold range. Blood from the same stock

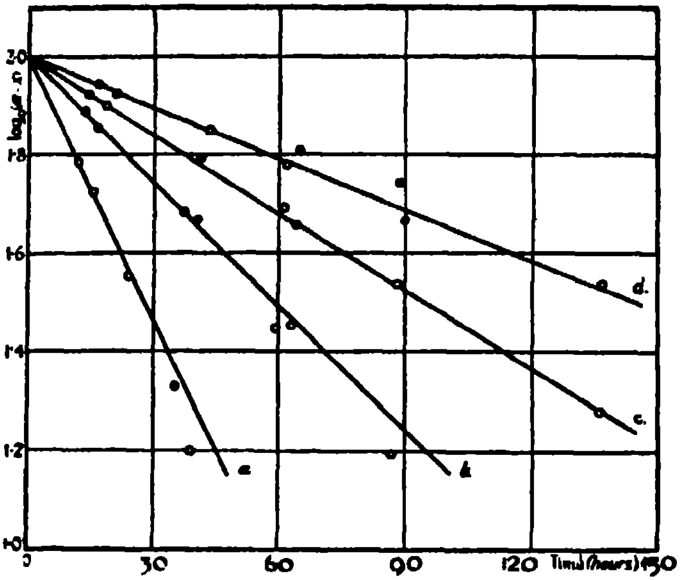

FIG. 1.—The rate of oxidation of hæmoglobin at (a) p_H 5·70; (b) p_H 6·29; (c) p_H 6·67 (d) 7·04.

solution was used in both experiments. The results are given in Table II and fig. 2. Four values from an experiment with a different sample of blood are included, in this case the total phosphate concentration was 0·6 M., the p_H 6·29.

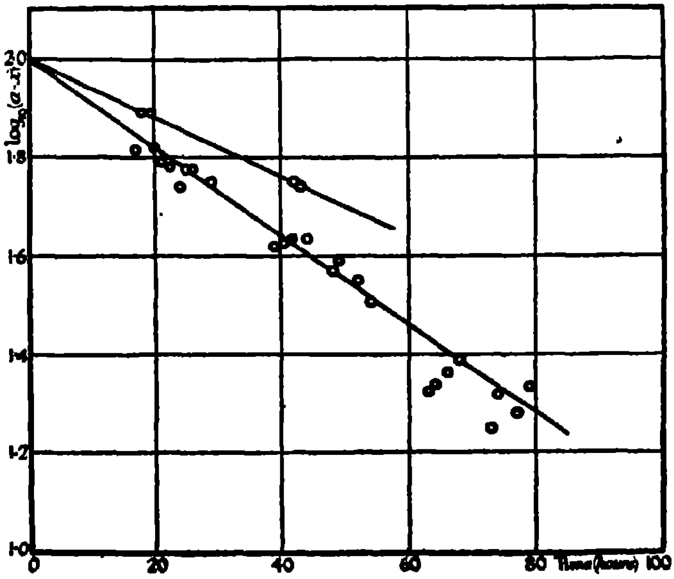

FIG. 2.—The rate of oxidation of hæmoglobin in solutions of different concentrations.

Table II.

0·2 M. total phosphate, p_H 6·29, 25° C.

Initial hæmoglobin.	Time.	Hæmoglobin.	$\log_{10}(a - x)$.
vols. per cent.	hours	vols. per cent.	
1·91	24	1·05	1·740
	48	0·71	1·570
	73	0·34	1·251
3·81	25	2·28	1·777
	49	1·48	1·589
	74	0·80	1·322
5·72	26	3·40	1·774
	52	2·03	1·550
	77	1·09	1·280
7·62	29	4·30	1·752
	54	2·44	1·505
	79	1·64	1·333
1·70	17	1·11	1·815
	39	0·71	1·621
	63	0·36	1·326
3·40	20	2·24	1·819
	40½	1·43	1·624
	64	0·74	1·338
5·10	21	3·15	1·791
	41½	2·20	1·635
	66	1·18	1·364
6·80	22½	4·08	1·778
	44	2·93	1·634
	68	1·67	1·390

0·6 M. total phosphate, p_H 6·29, 25° C.

Initial hæmoglobin.	Time.	Hæmoglobin.	$\log_{10}(a - x)$.
vols. per cent.	hours	vols. per cent.	
4·43	18	3·44	1·890
	42	2·49	1·750
8·86	19½	6·85	1·888
	43	4·87	1·740

Within the limits of the rather large experimental errors the monomolecular relation appears to hold in the range of concentration studied. The scattering of the points in the figure (chiefly below $\log_{10}(a - x) = 1·4$, *i.e.*, after 75 per cent. of the hæmoglobin has been oxidised) is probably not real, for the greatest deviations occur with the very dilute blood solutions.

Values of the velocity constant over the range of p_{H} 5·70–7·04 were determined at 25° C. with two further samples of blood. The three sets of values are given in Table III. The velocity constants, given by the equation

$$k = \frac{2 \cdot 303}{t} \log_{10} \frac{a}{a - x}$$

were obtained graphically, times being expressed in hours.

Table III.—0·2 M. total phosphate, 25° C.

p_{H}.	5·70.	6·29.	6·67.	7·04.	x.
$k \times 10^3 =$	4·11	1·97	1·21	0·79	0·54
$k \times 10^3 =$	3·11	1·56	0·95	0·58	0·54
$k \times 10^3 =$	2·22	0·94	0·60	0·34	0·61

The rates of oxidation were reproducible using the same stock solution of blood, but it will be seen that there is considerable variation between the three samples. The following values of k given by eight different samples may be quoted :—

0·2 M. total phosphate
p_{H} 6·29.
25° C.

$$k \times 10^3 = 0 \cdot 94,\ 1 \cdot 03,\ 1 \cdot 52,\ 1 \cdot 54,\ 1 \cdot 56,\ 1 \cdot 68,\ 1 \cdot 97,\ 2 \cdot 07.$$

It is known that the oxidation of hæmoglobin is very sensitive to catalysts and it seems probable that these differences are due to varying amounts of some catalyst in the blood. The general relation between p_{H} and k, however, appears to have the same form in each case. In fig. 3 values of $\log_{10} (k \times 10^3)$ from Table III are plotted against p_{H}. The points lie on straight lines, two of the lines are parallel and the slope of the third is not very different. Since

$$p_{\mathrm{H}} = m \log_{10} (k \times 10^3) + c,$$

it follows that the relation between the velocity constant and hydrogen-ion activity $a_{\mathrm{H}}+$ is given by an equation of the form

$$a_{\mathrm{H}}^{x} + = \text{constant} \times k.$$

Values of the power x are given in Table III.*

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* —x is the reciprocal of the tangent of the angles made by the straight lines in fig. 3 with the abscissæ.

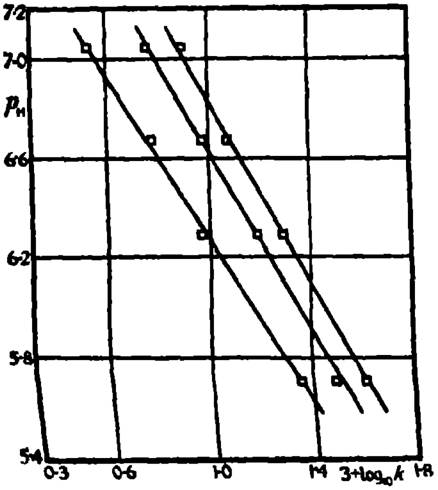

Fig. 3.—The relation between velocity constant and p_H.

The Temperature Coefficient of the Reaction.

The rate of oxidation was measured at 15° and 25° C. using the same stock solution of blood. The results are given in Table IV. The values of total and denatured pigment at both temperatures showed no significant change during the course of the reaction.

Table IV.—0·2 M. total phosphate, 25° C.

p_H.	Time.	Hæmoglobin.
	hours	vols. per cent.
5·70	0	5·30
	17	3·61
	41	2·04
6·29	0	5·30
	18	4·25
	42	3·50
6·67	0	5·30
	19½	4·66
	43½	3·97
7·04	0	5·30
	22	4·98
	45	4·51
5·70	0	4·51
	19	2·95
	43	1·70
	67	0·93

Table IV.—(continued).

p_H.	Time.	Hæmoglobin.
	25° C.	
	hours	vols. per cent.
6·29	0	4·51
	21	3·69
	44	2·91
	68	2·26
6·67	0	4·51
	22	3·93
	45	3·50
	69	3·01
7·04	0	4·51
	25	4·19
	49	3·84
	73	3·53
	15° C.	
5·73	0	5·30
	42	4·20
	113	2·83
	161	2·18
6·32	0	5·30
	43½	4·81
	114	3·93
	162	3·58
6·70	0	5·30
	46	5·01
	115	4·38
	163	4·11
7·07	0	5·30
	49	5·06
	118	4·68
	166	4·51
5·73	0	3·88
	64	2·70
6·32	0	3·88
	65	3·25
6·70	0	3·88
	66	3·55
7·07	0	3·88
	69	3·72

The velocity constants (obtained graphically) and the temperature coefficients are given in Table V. The ratio $k_{25°}/k_{15°}$ (neglecting the small p_H difference at the two temperatures) is approximately 4 except at p_H 7·06 (18° C.). At

15° C. the amount of pigment oxidised is relatively small, especially in the more alkaline solutions. The velocity constants at this temperature are therefore only approximate and the deviations from the most accurate value of the temperature coefficient, 4·05 at p_{H} 5·72 (18° C.), are probably not real.

Table V.

15° C.		25° C.		$k_{25°}/k_{15°}$.
p_{H}.	k.	p_{H}.	k.	
5·73	$5\cdot48 \times 10^{-3}$	5·70	$2\cdot22 \times 10^{-3}$	4·05
6·32	2·38	6·29	0·94	3·95
6·70	1·41	6·67	0·60	4·26
7·07	0·97	7·04	0·34	3·51

Two corrections should be applied to these values of the temperature coefficient (1) for the difference in p_{H} at the two temperatures (2) for the different concentration of dissolved O_2 at the two temperatures. The p_{H} correction was calculated from the equation

$$\alpha^{0\cdot61}_{\mathrm{H}} + = \text{constant} \times k,$$

and amounts to a decrease of 4 per cent. Neill and Hastings (1925) have shown that the rate of oxidation increases with decreasing oxygen pressure over a wide range but there is insufficient data for the calculation of the oxygen concentration correction. The effect is in the same direction as that of the p_{H} correction, a very rough estimate gave a decrease lying between 2 and 15 per cent.

The Influence of Phosphate Concentration.

The work of Cohn (1927) makes it possible to prepare buffer mixtures of the same p_{H} and differing concentrations of total phosphate. The rate of oxidation has been measured at 25° C. and p_{H} 6·29 in solutions of concentration 0·2, 0·6, 0·8 and 1·0 M. total phosphate. The values of $[\mathrm{HPO_4}^{-}]/[\mathrm{H_2PO_4}^{-}]$ given below were obtained by interpolation from Cohn's data.

p_{H} 6·29 (25° C.).

Total phosphate (M.)	0·2	0·6	0·8	1·0
$\dfrac{[\mathrm{HPO_4}^{-}]}{[\mathrm{H_2PO_4}^{-}]}$	$\dfrac{0\cdot300}{0\cdot700}$	$\dfrac{0\cdot375}{0\cdot625}$	$\dfrac{0\cdot395}{0\cdot605}$	$\dfrac{0\cdot405}{0\cdot595}$

The method of analysis of hæmoglobin and total pigment in the heavily buffered mixtures was modified slightly. The lactic acid concentration of the K_3FeCy_6 reagent was increased* and 2·5 N. NaOH was used for the absorption of CO_2. The volume of reagents used was the same, but the time of shaking with CO was increased to 5 minutes. When the appropriate corrections were used the same results were obtained with mixtures containing equal volumes of blood but different concentrations of total phosphate. The values for undenatured pigment in mixtures above 0·2 M. phosphate were somewhat lower than those for total pigment, but as the discrepancy did not change greatly during the reaction it was assumed that cathæmoglobin was not formed.

The results of experiments with the same sample of blood are shown graphically in fig. 4. Increase of phosphate concentration from 0·2 to 0·6 M. increased the rate by 19 per cent., above this concentration no further effect was observed. The values of k were :—

0·2 M. total phosphate	$k = 1{\cdot}54 \times 10^{-2}$
0·6–1·0 M. total phosphate	$k = 1{\cdot}83 \times 10^{-2}$

An unknown fraction of the increase must be attributed to the decreased concentration of dissolved oxygen.

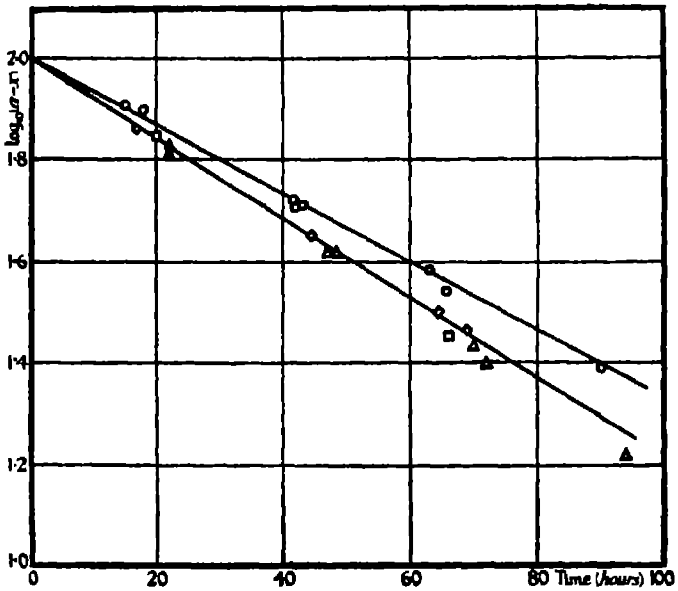

FIG. 4.—The rate of oxidation of hæmoglobin at p_H 6·29 in solutions of phosphate of concentration : (○) 0·2 M. (◇) 0·6 M. (□) 0·8 M. (△) 1·0 M.

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* The reagent used had the following composition :—20 grams K_3FeCy_6, 47 c.c. lactic acid (sp. gr. 1·21), 65 c.c. water.

It was found previously (1930) that a large addition of NaCl to blood diluted in a phosphate buffer greatly increased the rate of oxidation. Table VI gives the results of two experiments with NaCl-phosphate mixtures.

Table VI.—0·2 M. total phosphate; $[HPO_4^{=}]/[H_2PO_4^{-}] = 3/7$, 25° C.

Concentration of added NaCl (M.).	k.	Per cent. increase in rate.
0	$1 \cdot 54 \times 10^{-3}$	—
0·68	3·20	108
1·71	9·48	516

The acceleration of the rate is very marked. If the salt effect of NaCl is similar to that of phosphate the increases in Table VI must be attributed mainly to the decrease in p_H caused by the large addition of NaCl.

The Effect of Toluene.

With high concentrations of NaCl there is no danger of bacterial contamination of hæmoglobin solutions. It is possible in such a system to determine whether the presence of toluene has any effect on the rate of oxidation. The decrease in hæmoglobin concentration was determined in two mixtures of the same composition except that in one toluene was present (in the amounts previously described) and in the other toluene was absent both in the mixture and the current of air. The analyses were not done in duplicate and values of total and undenatured pigment were not determined. The time of shaking with CO in the analyses was 5 minutes.

Within the experimental error the reaction was monomolecular (Table VI) and the presence of dissolved toluene had no effect on the rate of oxidation. Bacterial action would also be absent in very concentrated buffer mixtures. In an experiment with a 1·0 M. total phosphate buffer of p_H 6·29 the agreement between the rate of oxidation of hæmoglobin with and without toluene was very good.

If a considerable amount of liquid toluene was present during the reaction an interesting effect was observed. The rate was always initially the same as that in a mixture containing only dissolved toluene, but relatively the rate increased as the reaction progressed. Fig. 5 shows graphically the rate of oxidation at p_H 6·29, total phosphate concentration 0·2 M. (1) with the usual amount of toluene (2) after addition of 1·0 c.c. toluene to 50 c.c. of the mixture.

The results in the presence of excess toluene suggest a reaction of the autocatalytic type. It was thought that denaturation of hæmoglobin might take place slowly at the toluene-liquid interface and the cathæmoglobin so formed catalyse the oxidation, but the unchanged values of total and undenatured pigment did not support this view. If cathæmoglobin or hæmatin was responsible the amount present was less than 0·1 vols. per cent. It is possible that some other substance changed or denatured at the interface may have acted as catalyst.

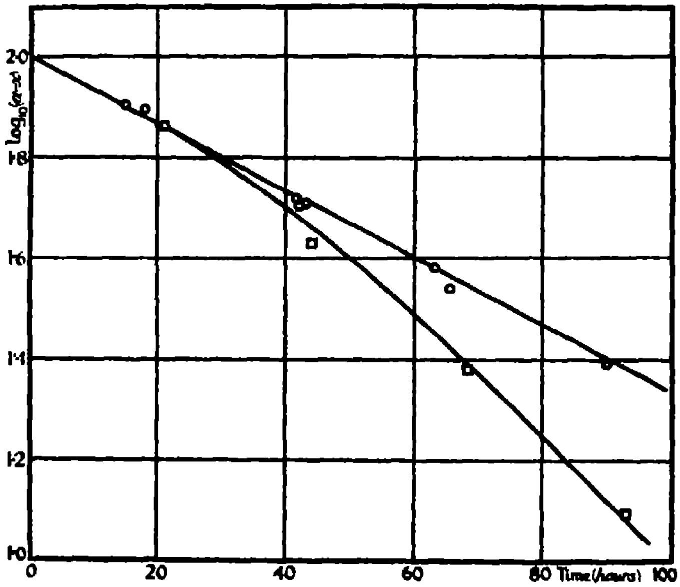

Fig. 5.—The rate of oxidation of hæmoglobin in the presence of : (○) a minimum amount of toluene. (☐) Excess of liquid toluene.

Discussion.

Conant and his co-workers (1923, 1924-25, 1926, 1928) have shown that reduced hæmoglobin and methæmoglobin form a reversible oxidation-reduction system of similar type to the ferrocyanide-ferricyanide system. The change of hæmoglobin to methæmoglobin involves a valence change of the iron atom and requires one hydrogen equivalent of oxidising agent per gram-atom of iron in the hæmoglobin molecule. The action of oxygen appears to be rather abnormal (as in several other oxidations with molecular oxygen) and Conant and Fieser (1924-25) have tentatively suggested the following schematic equation (neglecting the ionisation of the hæmoglobin):

$$\underset{\text{Reduced hæmoglobin}}{2H_3(Hb)} + O_2 \rightarrow \underset{\text{Methæmoglobin}}{2H_2(MHb)} + H_2O_2$$

where $H_3(Hb)$ contains 1 gram-atom of iron. As Adair (1925) and Svedberg (1926, 1927) have shown that the hæmoglobin molecule contains 4 gram-atoms of iron the equation can be written

$$[H_3(Hb)]_4 + 2O_2 \rightarrow [H_2(Hb)]_4 + 2H_2O_2.$$

If the kinetics of the reaction are given by this equation or a similar one the rate should be monomolecular with respect to the hæmoglobin concentration. The probability that some catalyst in the blood solution influenced the rate of oxidation in the present experiments makes it impossible to assume without further evidence that the observed monomolecular rate can be explained on this simple basis. Further work with solutions of pure hæmoglobin is required.

The higher rate of oxidation of hæmoglobin in the more acid solutions is another example of the analogy between the properties of the hæmoglobin-methæmoglobin and ferrocyanide-ferricyanide systems pointed out by Conant (1923). The ferrocyanide ion is comparatively stable to oxygen, but Baudisch and Davidson (1926-27) have shown that the substituted ferrocyanide, penta-cyanoammine-ferroate ion, is oxidised by oxygen to the ferri-ion and that the rate is inversely proportional to the p_H. Simple ferrous iron, on the other hand, is rapidly oxidised by oxygen in alkaline solution but is comparatively stable in acid solution.

Summary.

(1) The decrease of hæmoglobin concentration in diluted blood containing dissolved oxygen was due to the formation of methæmoglobin.

(2) The oxidation of hæmoglobin to methæmoglobin was, within the experimental limits, monomolecular with respect to the concentration of hæmoglobin.

(3) The rate of oxidation varied in different samples of blood.

(4) Decrease of p_H increased the rate of oxidation. The rate was directly proportional to a power of the hydrogen-ion activity. With three different bloods the value of the power was 0·54, 0·54 and 0·61.

(5) The uncorrected temperature coefficient was approximately 4 over the

p_H (18° C.) range 5·72–7·06. The correction for the difference in concentration of dissolved oxygen at the two temperatures was unknown.

(6) Increase of the concentration of phosphate at p_H 6·29 from 0·2 to 0·6 M. increased the rate of oxidation by 19 per cent. Increase from 0·6 to 1·0 M. had no further effect. An unknown fraction of the increase must be attributed to a decrease in the concentration of dissolved oxygen.

(7) Dissolved toluene had no effect on the rate of oxidation in concentrated salt solutions. The presence of considerable amounts of liquid toluene changed the course of the reaction.

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*Therapeutic Interference caused by Isomerides of Trypanocidal Styryl Quinoline Derivatives.**

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The quaternary salts of the styryl quinolines (Browning, Cohen, Ellingworth and Gulbransen, 1926, 1929) which possess active trypanocidal properties *in vivo*, can usually be prepared in two ways—(*a*) by condensation in presence of piperidine of the quaternary salt of the quinaldine derivative with the aldehyde (method I), or (*b*) by forming the quaternary salt by the addition of methyl sulphate or alkyl halide subsequent to condensation with the aldehyde, using zinc chloride as condensing agent (method II). It was found that certain compounds synthesised by method II were strikingly deficient in trypanocidal action as compared with their isomerides prepared by method I; there were also marked differences in chemical and physical properties. The present investigation elucidates these differences.

Biological Part.

2(*p*-amino styryl) 6 acetylamino quinoline methosulphate (the corresponding methochloride is substance No. 8) as prepared by method I, like No. 8, has a powerful curative action when administered subcutaneously to mice infected with *T. brucei*. When treatment was delayed until parasites were somewhat more abundant in the blood than in the usual therapeutic experiments, a dose of 1 : 3000 per 20 grams body weight† usually led to complete cure (maximum tolerated dose 1 : 750). On the other hand, the isomeride prepared by method II (maximum tolerated dose 1 : 300) when administered under similar conditions in doses of 1 : 300 or 1 : 500 usually produced only a trace of action, life being prolonged for from one to several days beyond that of the untreated controls which died about 72 hours after inoculation. Sometimes the largest dose had no effect; occasionally life was prolonged for 8 or 9 days. In the latter case the trypanosomes increased considerably in number in the blood for several days after treatment, they then gradually disappeared and remained absent for 2 or 3 days, but they re-appeared again and increased progressively, causing death after an acute course. Not merely was this substance deficient in trypanocidal action, but also it produced therapeutic interference (Browning and Gulbransen, 1922, 1927); that is to say, when administered along with the active isomeride it diminished or practically annulled the trypanocidal action of the latter. In this instance the effect was produced by injecting a mixture of the two substances in solution. In one series two animals which received doses of 1 : 3000 of product I showed no parasites in their blood over a period of observation extending to 97 days; but of those which received in addition 1 : 500, 1 : 1000 or 1 : 2000 of product II (2 animals received each dose) 3 died along with the controls and only 1 survived to the eighth day. On diminishing the amount of the interfering substance the results varied with the individual animal, but even 1 : 20,000 or 1 : 30,000 of product II might produce a marked interference when associated with 1 : 3000 of product I, so that parasites increased in the blood for several days, then diminished and disappeared for 1 or 2 days, again returning and increasing progressively until death resulted. Where the interfering substance did not prevent cure, the parasites persisted for several days in the blood, often increasing before their final disappearance. This increase has also been noted by Schnitzer and Rosenberg (1926) as a manifestation of slight interference. With as small an amount of substance II as 1 : 100,000 along with 1 : 3000 of substance I, such delay was evident.

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* The work reported in this communication was done with the support of the Medical Research Council.

† The doses mentioned refer to the dilution of which 1 c.c. was administered to a mouse weighing 20 grams; for mice of other weights the amounts given were in proportion to the body weight; all the animals used weighed from 18 to 22 grams.

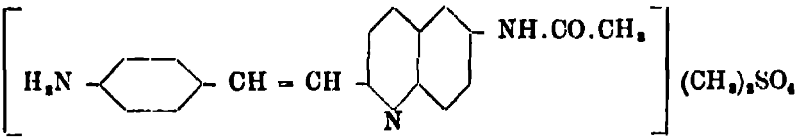
In the case of 2(*p*-acetylamino styryl) 6 aminoquinoline methochloride (No. 87) prepared by method I (tolerated dose 1 : 2000) cure was effected by doses of 1 : 5000 and sometimes by 1 : 7500 and 1 : 10,000. With a product prepared by method II (tolerated dose 1 : 750) 1 : 800 had sometimes only slight action; and even where the blood eventually became free from trypanosomes after a dose ranging from 1 : 800 to 1 : 2000, they increased markedly and persisted for about 3 days before disappearing. From the latter product a fraction separated by crystallisation from dilute alcohol had the chemical and physical characters of the substance prepared by method I and its toxicity and trypanocidal action corresponded with those of the latter. Thus, method II led to the formation of both isomerides.

2(*p*-acetylamino styryl) 6 acetylaminoquinoline methosulphate (No. 89; No. 88 is the corresponding methochloride) was formed by both methods of preparation, according to the chemical and physical properties of the products; the toxicity and trypanocidal action of the two were practically the same.

In connection with the investigation of relationships between chemical constitution and chemotherapeutic action, great importance attaches to the above observations on account of the possible source of error which they elucidate. It is demonstrated that, as in the case of No. 87, a method of synthesis may give rise to a mixture of isomerides the one of which is therapeutically active, whereas the other is not merely relatively inactive but, in addition, has the property of interfering with the chemotherapeutic effect of the former. The low concentration in which the inactive isomeride may give rise to therapeutic interference (1 : 100,000 in the case of the isomeride of the methosulphate corresponding to No. 8) indicates the necessity for exercising great care in drawing conclusions as to the chemotherapeutic efficacy of substances investigated in an impure state.

Chemical Section.

The substance 2(*p*-amino styryl) 6 acetylamino quinoline methosulphate (the methosulphate corresponding to No. 8 or 8S) of the formula



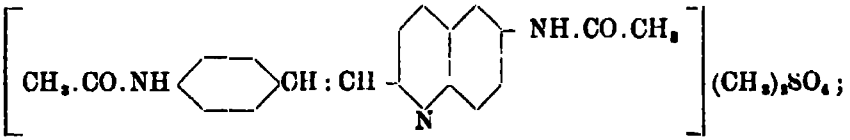
was prepared by the two methods described above, and although the compounds are isomeric and contain the same content of sulphur (S found for I = 7·11 per cent.; II = 7·2 per cent.; $C_{21}H_{23}O_5N_3S$ requires S = 7·4 per cent.) the chemical and physical properties were observed to be very different; the differences are tabulated as follows:—

I.	II.
Large bright red plates or needles, m.p. 270° (decomp.). Boiling with ammonia produced no change, the original substance being recovered by salting out with sodium sulphate.	Microscopic dark red crystals, m.p. (280° (decomp.) Boiling with ammonia precipitated the original base. This was proved by mixed m.p.'s and conversion by acetylation into 2(*p*-acetylamino styryl) 6 acetylamino quinoline.

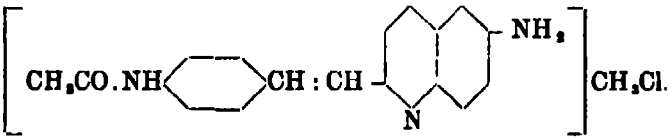
I.	II.
Hydrolysis with concentrated hydrochloric acid and neutralisation with ammonia gave 2(*p*-amino styryl) 6 amino quinoline methochloride, identical with a pure synthetic specimen.	Hydrolysis with concentrated hydrochloric acid and subsequent neutralisation with ammonia gave 2(*p*-amino styryl) 6 amino quinoline, proved by comparison with a pure specimen and conversion into the above diacetyl base on acetylation.

The only group which is open to attack by the methyl sulphate, other than the quinoline nitrogen is the free amino group of the benzene nucleus, but this is negatived by the fact that the unsubstituted diamino derivative is re-formed on hydrolysis.

In order to determine whether the observed differences depended on the free amino group, the following two substances were prepared by the two methods, the one containing no free amino group and the other an amino group in the quinoline nucleus.



No. 89.



No. 87.

In the case of No. 89 no differences were observed; both products crystallised from methyl alcohol in yellow needles, m.p. 295° (decomp.), gave the same mixed melting point and contained the same content of S. S found for I = 6·9 per cent.; II = 6·8 per cent. $C_{23}H_{25}O_6N_3S$ requires S = 6·8 per cent.

In the second case (No. 87) the methosulphate of the II product was prepared and on crystallisation from alcohol about 10 per cent. of the substance was separated and differed from the remainder in containing material which was precipitated by ammonia, although it had the same content of sulphur. S = 7·6 per cent.; $C_{21}H_{23}O_5N_3S$ requires 7·4 per cent. The dried substance had a green metallic lustre. The remaining 90 per cent. on evaporation deposited red crystals which were stable to the action of ammonia and gave on analysis $S = 7 \cdot 4$ per cent.; $C_{21}H_{23}O_5N_3S$ requires 7·4 per cent.

After conversion to the chloride it appeared to be identical with the methochloride prepared by method I, being stable to ammonia and yielding the diamino methochloride on hydrolysis with hydrochloric acid. It crystallised in red needles from alcohol, identical with the pure I product as determined by crystalline form, melting-point and mixed melting-point.

It follows from the above observations that in the case of No. 88, and to a small extent in the case of No. 87 prepared by the method II, the methyl sulphate does not form a true quaternary salt, but a loose molecular complex, which is easily detached by the action of ammonia or by hydrolysis with hydrochloric acid.

2(p-*nitro styryl*) 6 *acetylamino-quinoline.*—*p*-nitrobenzaldehyde (20 grams) and 6 acetylamino-quinaldine (26·5 grams) were melted together in a metal bath at 140°–150°. Powdered zinc chloride (2 grams) was added and the mixture maintained at this temperature for three-quarters of an hour. Rapid condensation occurred and the product solidified with evolution of steam. The yield after crystallisation from benzene was 33 grams. It crystallised from pyridine in yellow, lenticular crystals, m.p. 274°–278°. Found N, 12·90 per cent.; $C_{19}H_{15}N_3O_3$ requires N, 12·62 per cent. The high percentage of nitrogen is probably due to slight hydrolysis, for on boiling with a little acetic anhydride the percentage of N was 12·65.

2(p-*amino styryl*) 6 *acetylamino-quinoline.*—The nitro compound (33 grams) was suspended in 350 c.c. 90 per cent. acetic acid, steam-bath and iron filings (30 grams) slowly added with frequent shaking. The product was filtered, made alkaline with ammonia, filtered and the precipitate extracted with alcohol containing ammonia. On concentration of the alcoholic solution, the base crystallised from alcohol in small square plates, m.p. 212°–214°. After two re-crystallisations from alcohol it melted at 224°–226°; yield 18 grams. Found N, 14 per cent.; $C_{19}H_{17}N_3O$ requires N, 13·9 per cent.

2(p-*acetylamino styryl*) 6 *acetylamino-quinoline.*—The above amino compound (5 grams) was heated on the steam-bath with acetic anhydride (25 c.c.) and fused sodium acetate (1 gram) for 1 hour. The product was filtered, washed with water and dilute ammonia. It was boiled up with absolute alcohol, filtered and allowed to crystallise. The yield was 4·5 grams of pale yellow amorphous powder. This was boiled with half a litre of absolute alcohol which left a residue, m.p. 304°–306°, whilst the solution deposited 2·5 grams, m.p. 312°–314°, which crystallised in long thick colourless needles. Found N, 12·3 per cent.; $C_{21}H_{19}N_3O_2$ requires N, 12·2 per cent.

2(p-*acetylamino styryl*) 6 *amino-quinoline.*—Acetylaminobenzaldehyde (5 grams) and 6 amino-quinaldine (5 grams) were heated in a metal bath at 140°–145°. The mixture slowly melted to a thin liquid whilst steam was evolved and solidification occurred. Heating was then continued for another half-hour. The product was crystallised from alcohol and forms short colourless needles, m.p. 216°–218°; the yield was 5·2 grams. Found N, 13·9 per cent.; $C_{19}H_{17}N_3O$ requires 13·9 per cent.

2(p-*amino styryl*) 6 *amino-quinoline.*—By boiling the above acetylamino compounds with excess of concentrated hydrochloric acid for 2 hours and then adding excess of ammonia, a precipitate was formed which crystallised from dilute alcohol in yellow needles, m.p. 241°–242°. Its solution in toluene was yellow with a deep blue fluorescence.

2(p-*amino styryl*) 6 *acetylamino quinoline, methosulphate.*—This substance is the methosulphate corresponding to the methochloride (No. 8) and may be denoted by No. 8S. It was prepared by method II, as follows: Theoretical quantities of the base and dimethyl sulphate were heated in nitrobenzene solution on the steam-bath for 10 minutes. On cooling, a red crystalline product separated and was filtered, washed with nitrobenzene and ether and boiling absolute alcohol and finally crystallised from 90 per cent. alcohol. It melted at 280° with decomposition. A mixed melting-point with 8S prepared by method I gave 250°–260°. Found S, 7·2 per cent.; $C_{19}H_{17}N_3O, (CH_3)_2SO_4$ requires S, 7·4 per cent. On boiling the above (8S, method II) with excess of ammonia for 10 minutes it was completely precipitated and the product was proved by a mixed melting-point and by conversion into the *p*-acetylamino derivative to be the original 2(*p*-amino styryl) 6 acetylamino quinoline.

2(p-*acetylamino styryl*) 6 *acetylamino quinoline, methosulphate* (No. 89).—The base (1·5 grams) was dissolved in boiling nitrobenzene (100 c.c.) and dimethylsulphate (0·7 c.c.) added and the mixture allowed to cool slowly. The product which separated was filtered and extracted with 300 c.c. of methyl alcohol. The residue formed long, yellow needles, m.p. 288°–290° (decomp.). Found S, 6·8 per cent.; $C_{21}H_{19}N_3O_2$ requires S, 6·8 per cent. Boiling with ammonia for some minutes produced no apparent change (no precipitate) either with No. 89 prepared by method I or II. On the other hand, hydrolysis with concentrated hydrochloric acid gave the same product, m.p. 270°–273° (decomp.) from both (2(*p*-amino styryl) 6 amino quinoline methochloride).

2(p-*acetylamino styryl*) 6 *amino-quinoline, methochloride* (No. 87).—This was prepared as above by method II, using theoretical quantities of the base and dimethylsulphate, which were heated for 10 minutes at 120°–130°. The product was extracted by boiling with alcohol and the residue crystallised from dilute alcohol. The alcoholic extract gave a product unstable to ammonia, whereas the dilute alcohol yielded a substance identical with No. 87 obtained by method I. A larger yield of the unstable compound was given when the preparation was made at 150°. The stable product was purified by dissolving in water and a little ammonia, boiling with charcoal, filtering and precipitating the methochloride with saturated salt solution. The melting-point and mixed melting-point with the methochloride made by method I were identical, namely 299°–300° (decomp.). Hydrolysis with hydrochloric acid gave the same product as 8 obtained by method I, namely 2(*p*-amino styryl) 6 amino quinoline methochloride of m.p. 270°–272° (decomp.).

Summary.

In the case of certain quaternary salts of styryl quinolines which possess active trypanocidal properties *in vivo*, *e.g.*, 2(*p*-amino styryl) 6 acetyl amino quinoline methosulphate, synthesis is effected by condensation in the presence of piperidine of the quaternary salt of the quinaldine derivative with the aldehyde. The isomeride prepared by the addition of methyl sulphate subsequent to condensation of the aldehyde, using zinc chloride as the condensing agent, leads to a compound which is not a true quaternary salt but a loose molecular complex. The latter substance is much less effective as a trypanocidal agent; further, when administered along with the former, it produces therapeutic interference, on testing for trypanocidal action in mice infected with *T. brucei.* In the case of 2(*p*-acetyl amino styryl) 6 amino quinoline methochloride, the second method leads to a mixture of the two isomerides which is deficient in trypanocidal action as compared with the quaternary salt prepared by the first method. Since the inactive isomeride may cause therapeutic interference when present in high dilutions along with the corresponding active compound, it appears in general that great care is therefore necessary in drawing conclusions as to the therapeutic efficacy of impure substances.

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The Relation of Glutathione to Cell Respiration with Special Reference to Hepatic Tissue.

By Sir F. Gowland Hopkins, P.R.S., and K. A. C. Elliott (Beit Memorial Research Fellow).

§ I.—Introductory.

It has been commonly assumed that one function of glutathione in the tissues (others have been suggested) is to act, in virtue of its sulphur groupings, as a carrier of hydrogen from reducing systems to molecular oxygen. This assumption has hitherto been based upon its behaviour when added to washed tissue preparations.

The observations of Meldrum and Dixon (1930), however, have shown that the tripeptide, after its separation from the tissues in crystalline form, while freely reduced by tissue preparations, undergoes oxidation by molecular oxygen only with the co-operation of two factors present as traces in most preparations of the substance; not alone iron (or copper) of which the necessity was long ago shown by Warburg and Harrison, but also some substance able to form catalytically active complexes with the metal. Unlike earlier, less pure, preparations the crystalline product when added to washed tissues entirely fails to produce a system capable of taking up oxygen. Especially studied in this relation was the effect of adding it to the "thermostable residue" from muscle (Hopkins and Dixon, 1922; Hopkins, 1921). Moreover, when a solution of the crystalline material in the reduced form is shaken up with such a tissue preparation and filtered the filtrate shows no oxygen uptake even when metal is added. The glutathione appears under this treatment to be inactivated. The above authors showed that this is because the tripeptide, and, apparently, also other peptides containing cysteine are, unlike cysteine itself, incapable of forming a catalytically active complex with iron or copper. On contact with a washed tissue residue the factor which forms such a complex may, unless present in appreciable amount, be wholly removed by adsorption. Meldrum and Dixon showed good, if not quite conclusive, reasons for believing that this factor is free cysteine of which the earlier preparations contained more, but which may be present in traces even in the crystallised preparations. Mason (1931) has since suggested that the substance is more probably cysteinyl-glycine.

The demonstration of this stability of the tripeptide towards oxygen after isolation made it desirable that its fate when present in fresh intact tissues should be followed during the course of survival respiration. Its behaviour while thus retaining its normal associations in the cell should then be displayed.

The results to be described, in so far as they refer to its oxidation, have been obtained chiefly from studies of liver tissue. The reduced (thiol) form of glutathione as it exists in the liver we have found to be readily oxidised to the disulphide form by molecular oxygen. We have also found that its oxidation during the survival respiration of the fresh tissue is associated always with a continuance of reduction processes, showing that the transport of hydrogen to oxygen is thus maintained, and indicating that the oxidation of the tripeptide has real metabolic significance. The quantitative importance of this influence, moreover, would seem to be far from negligible.

Although the anaerobic reduction of the disulphide form by the tissues has been often described, it seemed desirable in the circumstances to obtain further data concerning this reducing power by the use of whole tissues, because most of the existing evidence refers to the action of tissues previously extracted with water. Experiments with this end are briefly described in section 3 (*a*). Meldrum and Dixon found that even the purest preparations of crystallised glutathione are normally reduced by tissue preparations.

The main purport of the paper, however, is to describe events during aerobic survival respiration.

What our experiments have actually demonstrated is that in the course of these events there is sustained oxidation and reduction, respectively, of thiol and disulphide groupings in the tissue. The evidence which suggests that glutathione is the dominant, and possibly the only, substance involved in these changes is sufficient to justify the title of this paper and the special reference to glutathione throughout. The behaviour of sulphur groups as displayed in the experiments applies to the behaviour of those in glutathione. Until, however, the tripeptide can be isolated more quantitatively than at present the absence of other cell constituents carrying active sulphur groupings with similar behaviour ought not to be assumed. This matter receives further reference in the next section.

§ II.—EXPERIMENTAL METHODS.

(*a*) *Estimation of Reduced Glutathione.*

Tunnicliffe's method (1925) in which trichloracetic acid extracts of tissues are directly titrated with N/100 iodine, using sodium nitroprusside as an external indicator, has been widely used and has met with both approval and criticism. It involves, of course, the assumption that no substance in the extract reduces iodine at a rate at all comparable with that involved in the oxidation of the thiol to the disulphide form of glutathione. This assumption is, we believe, in a practical sense, justified. Towards the end of a titration when the concentration of the —SH group has become very low there is some tendency to add a disproportionate amount of iodine. It is best to take as the end-point the stage at which a very slight pink colour with nitroprusside is still to be observed. Owing to the delicacy of the colour reaction this corresponds to a very small amount of glutathione. Throughout the main range of titration we believe the error to be negligible. The best evidence for the essential adequacy of the method lies in the nature of our results as will be noted later, after the discussion of these.

Nevertheless, owing to the greater convenience of titration with an internal indicator, we made a number of comparisons of Tunnicliffe's method with other methods in which starch has been so used. The procedure described by Perlzweig and Delrue (1927) gave in our hands poor agreement. On the other hand, when we observed the conditions for titration recently defined by Kühnau (1931), by adding 20 c.c. of a 25 per cent. solution of potassium iodide to the solution *before* the iodine was run in, we reached good agreement, and in many of our later estimations we employed this method with results identical in character with those given by Tunnicliffe's method.

(*b*) *Isolation of Glutathione from Mammalian Livers.*

Pirie (1930) isolated crystalline glutathione from ox liver, and since then the pure substance has been several times prepared in this laboratory from mammalian livers. The preparations have yielded analytical data agreeing with theory, and have shown all the properties of the pure substance as obtained from yeast and other sources. The method described by one of us (Hopkins, 1929) is indeed one which provides a moderate yield of the tripeptide with quite remarkable ease. It is, however, difficult to obtain by its means a yield which is quantitative. The substance is actively adsorbed by most precipitates and perhaps especially by the metallic sulphides which have to be dealt with in the process as described. In the final precipitation by cuprous oxide equilibria are set up which tend to make the separation partial, and the degree of this interference seems to vary with the nature of the tissue employed. In the case of the liver, moreover, experience suggests that the proportionate yield may vary with the metabolic condition of the organ. The difficulty due to the fact that the cuprous compound redissolves when excess of copper has been added has been removed by Pirie's observation that oxidation of the excess of metal, by aeration of the solution, results in reprecipitation. Even when excess has been avoided however, precipitation is usually incomplete at this stage. These aspects of the method are being further investigated in the hope of improving the yield.

From ox and rabbit livers yields representing from 40 to near 60 per cent. of the content as indicated by titration of the original tissue have been obtained. These amounts were determined by weighing the copper compound as isolated; in each case this gave accurate figures on analysis.

That such yields are compatible with the whole thiol content of the tissue being due to glutathione is shown by the proportion recovered when known amounts of the substance have been first added to tissues. The nature of the experimental results described in the next section is such as to suggest that a uniform substance undergoes change. We have thus felt justified in speaking only of glutathione throughout this paper. It is almost certainly the dominant thiol compound, if not the only one, in the tissues. Clearly however, as we have already suggested, until a really quantitative method of isolation is available we cannot be sure there are no others. Our experience agrees with that of Bierich and Kalle (1928) in finding that no free cysteine can be detected in perfectly fresh livers.

(c) *Reduction Experiments.*

The method used was the following :—

The fresh tissue was chopped up, sampled, and its initial content of GSH determined by titration. About 5 gm. was weighed into each of a series of 100 c.c. Thunberg tubes. In one tube the tissue was covered with 30 c.c. of distilled water only. This was incubated with the others to serve as a control. Into each of the others oxidised glutathione, usually 50 mgm., was washed with the use of 30 c.c. water, and the mixture brought to p_H 7·6 with N/10 soda. A few drops of chloroform were added and the tubes were then evacuated and incubated for a defined period at 37° C. Each was then filled with 10 per cent. trichloracetic acid by opening the tap with the side tube immersed in the acid solution. The tissue residue was filtered off and twice extracted by grinding with sand and further amounts of trichloracetic acid. The titration of —SH was then carried out on the combined filtrates.

(*d*) *Oxidation Experiments.*

The same animals were employed as in the reduction experiments, namely, rabbits, oxen, sheep, dogs, cats and rats, but oxidation has been fully studied in hepatic tissue only. In the greater number of the experiments rabbit livers were employed. The rabbits were killed in the usual manner by a sharp hand blow on the back of the neck or, when blood-free livers were required, by severing the carotids. In the case of ox- and sheep-livers the material was dealt with while still warm from the slaughterhouse. Cats were killed instantaneously by pithing; dogs by the use of the "humane killer," and the rats by decapitation. The liver in every case was removed as quickly as possible, and immediately chopped fine with scissors. It was never ground up, destruction of hepatic cells being avoided. In the case of animals killed without bleeding the liver pulp as used contained, of course, a considerable proportion of blood; in other cases the residual blood was so far as possible removed by pressure. The presence or relative absence of blood, though affecting, of course, the total quantity of glutathione in the pulp made no difference whatever to the course of oxidation.

The finely chopped liver was thoroughly sampled, and lots, each of 10 gm., were quickly weighed into a series of wide-mouthed bottles of 200 c.c. capacity. 20 c.c. of mammalian bicarbonate—Ringer's solution at p_H 7·6 were added to each sample, and all the bottles were closed with well-fitting rubber stoppers. All were then packed into the carrier of a shaking machine and individual samples removed at determined (usually 2-hourly) intervals during the shaking, for treatment and titration. Since the liver tissue as prepared made a somewhat thick suspension vigorous shaking was employed; the machine making about four oscillations per second. That bottles of the size employed contained sufficient air to make the oxygen supply fully adequate throughout the course of oxidation was shown by control experiments in which the rubber stoppers were removed at half-hourly intervals. Fresh air was blown into each bottle, the stopper replaced and the shaking continued. This treatment had no effect whatever upon the rate of oxidation as otherwise observed.

The mechanical treatment had no indirect effect. Individual hepatic cells when examined microscopically after the shaking showed no marked disintegration, and in bottles evacuated and washed out with nitrogen the concentration of the —SH group in the tissue remained constant. Changes in p_H during the aeration were at most very small.

Each sample on removal was immediately treated with 40 c.c. of 10 per cent. trichloracetic acid and then ground up with sand (of which a minimum was used to avoid adsorption effects), filtered, and the residue re-extracted and washed on the filter till the filtrate gave no trace of colour with nitroprusside. The filtrate was then titrated either by Tunnicliffe's or Kühnau's method.

The simple technique involved in thus aerating the tissue as described and titrating successive samples gave entirely consistent results. Whenever duplicate estimations were made at any stage the agreement was close.

In the few experiments done at 37° C. the shaking apparatus was transferred to a hot room kept at that temperature. In no case was there any indication of significant bacterial growth during the period of an experiment. The fresh, aerated, hepatic tissue so long as survival respiration continues is highly resistant to invasion by organisms.

§ III.—EXPERIMENTAL RESULTS.

(*a*) *Reduction of GSSG by Tissues under Anaerobic Conditions.*

The data in the following tables show the amount of GSSG‡ reduced by a variety of tissues under uniform conditions during a standard period of 3 hours. They provide therefore a measure of the relative reducing power of any one tissue compared with that of others. A proportionately short period of observation was chosen in order to avoid all possibility of errors due to bacterial action.

In all cases the figures correspond to milligrammes of GSH per 1 gm. of untreated tissue. Column A in each case shows the amount initially present

Liver.

Ox.		Sheep.		Rabbit.		Rat.		Dog.		Cat.*	
A.	B.	A.	B.	A.	B.	A.	B.	A.	B.	A.	B.
4·3	6·0	3·9	4·1	3·1	2·5	3·1	1·3	1·0	1·5	1·6	2·5
1·6	5·1	2·0	3·3	3·1	2·3	1·6	1·8	1·9	1·2	2·0	1·8
1·5	3·6	1·6	3·9	2·9	2·2	1·6	1·8			1·4	2·5
		2·8	5·7	4·0	2·6					1·5	3·9
				1·8	3·0						
				4·4	3·0						
				2·1	2·4						
5·0†		4·4†		2·1†		1·6†		1·4†		2·7†	

* Time curves for cat liver showed that after 3 hours the reduction of —SS had not approached completion. (*See next section.*)

† Mean increase.

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‡ The symbols GSH and GSSG are for convenience, and according to established usage, employed for the reduced and oxidised form of glutathione throughout this paper.

Kidney.

Ox.		Sheep.		Rabbit.		Rat.	
A.	B.	A.	B.	A.	B.	A.	B.
1·6	3·3	1·1	5·8	1·5	4·1	0·8	4·7
1·5	2·8	1·6	7·2	1·1	4·0	1·0	5·0

Muscle.

Ox.		Sheep.		Rabbit.		Rat.	
A.	B.	A.	B.	A.	B.	A.	B.
0·75	2·05	0·5	1·1	0·5	1·1	0·25	1·35
0·5	1·8			0·75	1·35		
				0·9	0·6		
				0·4	1·0		
				0·2	1·2		

Brain.

Ox.		Sheep.		Rabbit.		Rat.	
A.	B.	A.	B.	A.	B.	A.	B.
0·5	1·3	0·9	2·8	0·4	2·4	0·25	1·65
0·9	2·4	1·0	1·4	0·6	1·65	0·4	1·4
0·9	1·0	0·2	1·4				

in the tissue; column B gives, not the final, but the *extra* amount produced by the reduction of the added GSSG which was always present in excess. The figures show, as the observations of others have shown, that for any one tissue individuals vary greatly with respect to the initial SH content. As regards the reserve reducing power the variation would seem to be less. This latter diminishes with diminishing size of the animal, at least in the series, ox sheep, rabbit, rat; a circumstance which bears upon facts to be presented in the next section.

The initial SH content of renal tissue is generally lower than that of hepatic tissue, but the former seems to possess more of the mechanisms concerned in the reduction of GSSG, though here the ox kidney is an apparent exception. The figures in the last table are for mixed brain tissue, but it was found that the grey matter shows a greater increase of GSH on incubating with GSSG than does white matter. This has been noticed by others.

The following table illustrates the effect of temperature on the rate of reduction in the case of livers from four individual rabbits. The figures refer to the extra GSH produced from added GSSG after incubation for 4 hours (milligrammes per 1 gm. tissue) :—

Liver No.	1	2	3	4
At 15°	2·9	4·3	1·2	2·3
,, 38°	3·9	6·7	3·3	4·8

Hopkins and Dixon (1922) showed that a thermostable residue from muscle, representing chiefly the tissue proteins containing a fixed SH group (Hopkins, 1925), can reduce GSSG. Thermolabile systems play, however, a large part in reduction by intact tissues, as after heating these at 52° C. before adding the GSSG the reducing power is much diminished.

The following figures are from experiments in which the reducing power of rabbit livers previously heated to 52° for 1 hour was compared with that of controls which had stood for an hour at 15°. They give as before the extra GSH produced by incubating with GSSG for 4 hours at 37° C. (milligrammes per 1 gm. tissue) :—

Liver No.	1	2	3	4
After 1 hour at 15° C.	2·9	5·8	3·3	4·4
,, 52° C.	2·2	2·0	2·2	2·7

All the observations under discussion in this section were made before the experiments of the next section were planned. They involved the use of evacuated Thunberg tubes left, according to custom, quiescent in the water-bath. As explained in the previous section, the rate of reduction was followed by titrating the contents of each of a series of tubes removed from the bath at measured intervals. Experience gained, however, during the progress of the later aerobic experiments showed that shaking the tubes increased the observed velocity of reduction to a noteworthy degree. In the case of tissues chopped with scissors but not further disintegrated, which was the form of material used in most experiments, the added GSSG reaches the surface of the cells only slowly, unless the containing vessel is shaken. Since, therefore, the observed velocity was affected by an accidental factor, and therefore lacked actuality, we refrain from reproducing the various curves obtained. One curve, however, relating to liver tissue, on which most of our survival respiration studies were made, is given in fig. A.

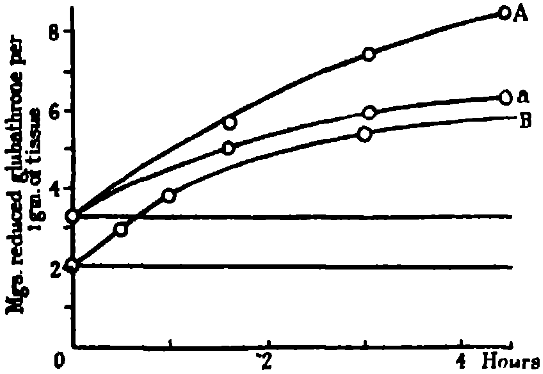


Fig. A.—Anaerobic reduction of added GSSG by sheep liver at 37° unshaken. A and B given by different individuals, (a) was given by the same liver as A after it had been passed through a very fine mincer instead of being chopped up. The horizontal lines show the initial concentrations of —SH.

Actual destruction of the tissue cells by grinding with sand was always found to slow the source of reduction, but not to an extent which would suggest that survival of the reducing mechanisms calls for histological integrity. The velocity of reduction was not significantly affected when Ringer solution replaced distilled water as the suspension fluid; but the use of phosphate buffer was found always to slow it.

(b) *The Course and Relations of the Oxidation of the Thiol Group.*

Attention may now be turned to the course of the disappearance of the thiol group when the liver preparations are aerated as described in Section 2 (*d*). Each "curve" or diagram in figs. 1–14 shows this course in the case of the liver of an individual animal. All are drawn to the same scale, and in every case each point on the curve was obtained by the titration of 10 gms. of tissue. Most of the curves are based upon the titration of some four samples only, one being the control while the others were removed from the shaker at 2-hourly intervals. In order to obtain fully representative samples we have preferred to use not less than 10 gm. of material; the liver of an individual rabbit may then yield no more than four or five samples. The data obtained will be found, however, to give ample evidence for the conclusions based on the experiments. In the case of an oxidative event occurring in complex relations any attempt to study its kinetics with closeness would be of little value, or, at any rate, without further knowledge, premature.

Rabbit Livers.—Our most numerous experiments have been made with these. The curves of fig. 1A show results obtained at room temperature (17°–18° C.) in the case of livers removed from rabbits which had been abundantly supplied with food (oats and carrots) for 24 hours preceding the experiment. All the results obtained under these conditions are assembled in fig. 1 no selection being made. The initial concentration of GSH varies widely in different livers, a familiar circumstance which will be discussed in another paper. In spite of

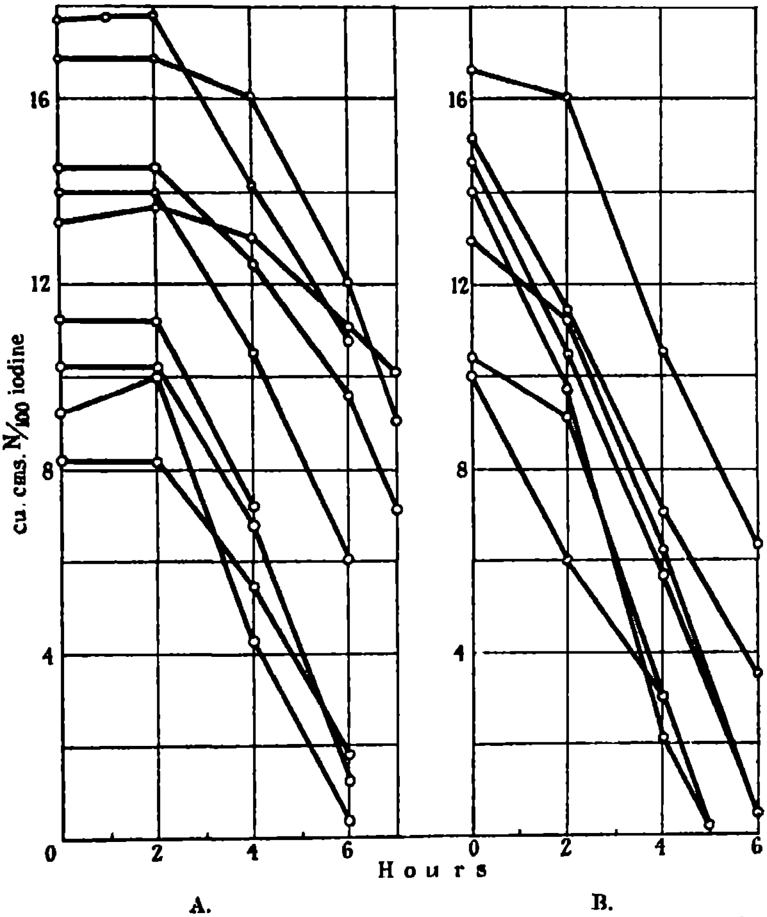


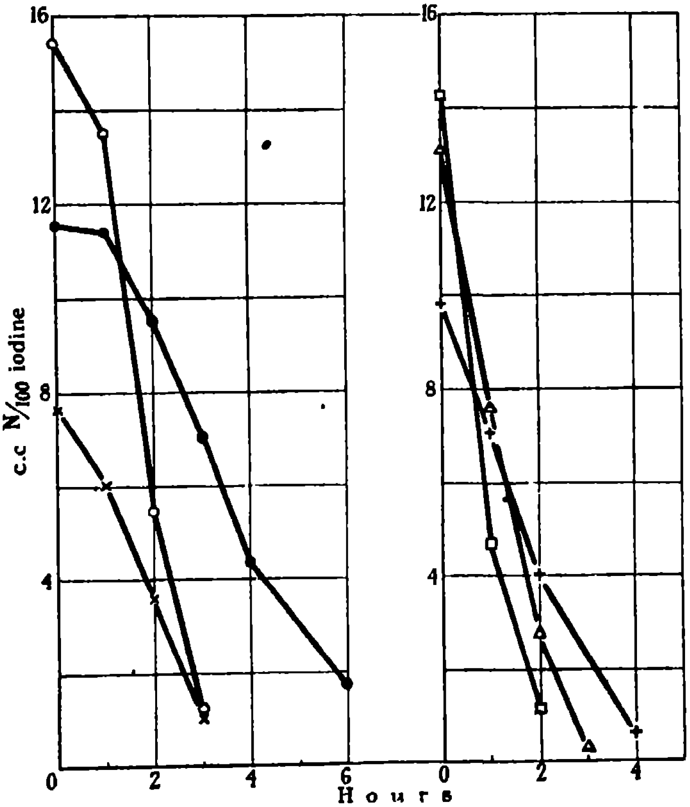
FIG. 1.—Shows progressive changes in the concentration of —SH during continuous aeration of liver tissue. Each " curve " or diagram was obtained from the liver of an individual animal. A, from livers of well-fed rabbits; B, from livers of fasting rabbits. Temperature 17° C. Each point on the curves was obtained by the titration of 10 gm. of tissue.

this variation one characteristic result is clear in every case. There is no appreciable change in the concentration of the —GSH during the first 2 hours of the vigorous aeration. If any change occurs it is rather a slight increase. That the transition from this period of apparent induction to a period of active oxidation appears in the diagrams as occurring exactly at the end of the second hour is due merely to our choice of intervals; but a period in which the concentration of GSH remains constant or nearly so is always followed by a rapid fall which starts quite early in the third hour of aeration. The constancy of this phenomenon in the case of well-fed rabbits is noteworthy. It will be seen that oxidative disappearance then proceeds, and, up to the sixth hour, its progress is approximately linear or may show a slightly increasing velocity.

At first sight it might seem possible to conclude from Meldrum and Dixon's observations that the initial delay in oxidation is due to the absence of any trace of free cysteine, which only appears later as the result of autolysis. A preliminary addition of cysteine to the tissue has, however, no effect at all on the subsequent course of oxidation. That the transition from slow to rapid oxidation is due to the appearance of any autolytic product, necessary for the formation of a catalytic iron-complex, is negatived by the result of the following experiment. The liver tissue of a rabbit was first shaken in the customary manner for 5½ hours and kept overnight in the ice chest. The whole was then divided into two equal parts, the —SH in one half estimated and the other half added to fresh liver tissue and the rate of the disappearance of —SH determined as in other experiments. There was no acceleration in the initial rate, but rather, after allowing for the small amount present in the previously aerated tissue, an actual rise in the concentration of —SH during the first 2 hours. This, as will be understood later, was due to reduction of the GSSG present in the added, previously aerated, liver.

It will be shown in what follows that the concentration of GSH is maintained at the beginning of the course *because oxidation in the freshly removed organ is then balanced by reduction.*

In fig. 1B are assembled curves obtained from the liver of animals kept without food (but given water) for 48 hours before the experiments were begun. It will be seen that the initial maintenance of GSH concentration is no longer seen when food has been thus withheld. It must be remembered that the contents of rabbits' intestines vary much during abstinence from food and some lack of uniformity might be expected. Nevertheless in four out of seven cases the concentration is seen to fall steeply from the very first without any indication of the delay characteristic of livers from well-fed rabbits. In two others the fall during the first 2 hours is marked enough. In one case only (uppermost curve) is there approximation to the curves of fig. 1, and in this case the animal had been deprived of food for 24 hours only. That this result is due to a fall in the velocity of reduction processes is a conclusion fully supported by other evidence. That it depends upon the disappearance, during fasting, of metabolites which act as hydrogen-donators seems equally clear. Before proceeding to discuss other evidence for this conclusion, however, we may draw attention to the results of a few experiments precisely similar in method to those just described but carried out at 37°. With the increased velocity in all processes at the higher temperature the concentration of hydrogen donators will diminish more rapidly, and though we made the first estimation after one hour instead of two this interval in some cases was too long for the complete balance between reduction and oxidation to be evident. The results obtained at this temperature from well-fed rabbits were irregular (fig. 2) but yield evidence fully



A. B.

FIG. 2.—Rabbits' Livers. Results similar to those of fig. 1, but obtained at 37° C. A, well-fed animals; B, fasting.

confirming those obtained at the lower temperature. In all cases the velocity of the oxidative disappearance is notably less during the first hour than during the second or subsequent hours, a circumstance not seen in the case of livers of animals previously deprived of food (fig. 2B). Indeed, a comparison of the curves of fig. 2 no less than those of fig. 1 show plainly enough the influence of the nutritional condition of the liver upon the apparent rate of oxidation.

Further evidence will now be advanced to show that processes involving the reduction of glutathione continue during the aeration of the surviving tissue and account for the apparent relative slowness of oxidation during the earlier periods of survival. With lessened velocity they continue indeed throughout the duration of the experiments, so that the observed rate of the disappearance of GSH is always a resultant of the opposed processes of reduction and oxidation. This is shown by the fact that if in the course of an aeration experiment such as those just described duplicate samples are removed at say, the fourth or sixth hour, one being titrated at once, while the second, after evacuation of the containing vessel at the pump, is allowed to stand anaerobic for a period of 2 hours or so before titration, a marked return rise in the concentration of SH is always observed.

In an organ with its circulation intact the oxidised form of glutathione may exist in dynamic equilibrium with the reduced form, but in an excised organ, even though the exposure of its constituent cells to lack of oxygen has been brief, the whole would seem to be reduced or nearly so. This is indicated by the circumstance that when the disulphide form is added to the tissue it promptly suffers reduction.

When indeed, as we are now to show, the concentration of the disulphide form is made adequate, the velocity of hydrogen transport is so much increased that even during a period of vigorous aeration the concentration of the thiol form rises instead of falling.

This is illustrated by the curves of fig. 3A. Of the pair with broken lines the lower is an ordinary curve furnished by the liver of a rabbit previously deprived of food. The upper is from the same liver, but to each sample yielding a point on the curve 100 mgm. of GSSG had been added before aeration. It is seen that after this addition the sharp fall in GSH characteristic of the first 2 hours of aeration is replaced by a slight rise. As might be expected from what has gone before the phenomenon is much more marked in the liver of a well-fed animal. This is shown by the unbroken curves of fig. 3A. The lower is a normal curve, the upper is from the same liver with GSSG added as just described. In spite of the aeration the customary plateau is replaced by a 60 per cent. increase in the concentration of GSH, as estimated at the end of the second hour. The rate of reduction during the first 2 hours was thus notably greater than that of oxidation. It should be noted, though the result is not shown in the figure, that the oxidation of the GSH (that derived from the GSSG added no less than that naturally present), was complete by

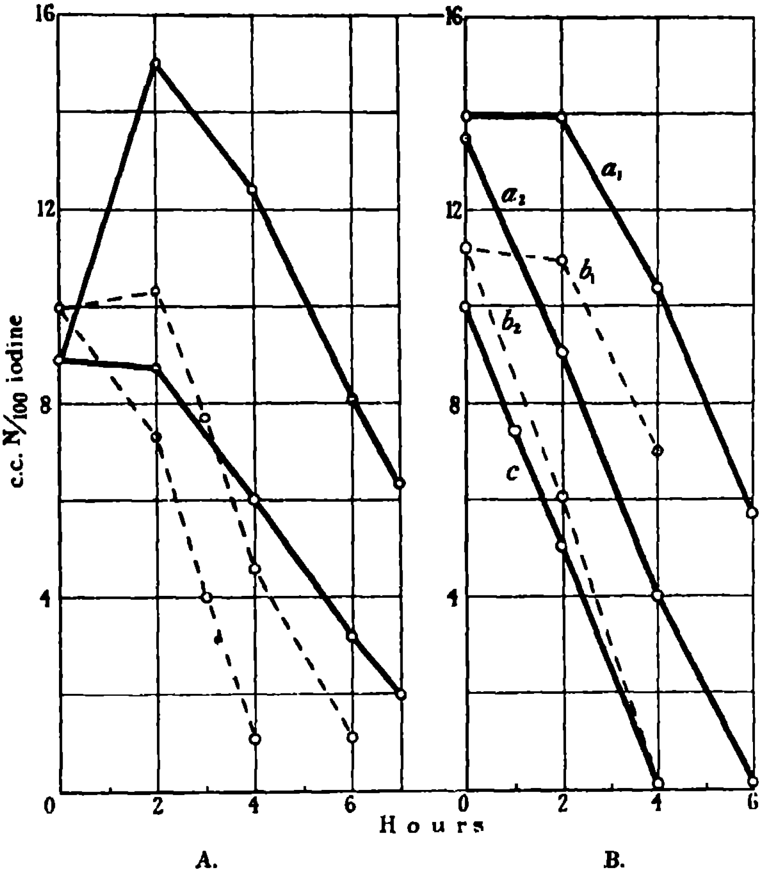


Fig. 3.—A. Showing effects of adding GSSG. *Continuous lines:* the lower curve shows the normal course of disappearance of —SH from liver of a well-fed rabbit; the upper curve was given by the same liver when 100 mgm. of GSSG had been added, before aeration, to each sample titrated. *Broken lines* showing results of a similar experiment with liver of fasting rabbit. Temperature 17° C. B. Effect of inactivating reducing systems in the liver. Curve a_1, normal course in liver tissue of well-fed rabbit; a_2, linear fall in concentration of —SH in same tissue previously heated to 50° C. for 1 hour; b_1 and b_2, a pair of similar curves from liver of a second individual; *c*, from liver of a third individual, linear fall after previous heating to 50° C. Temperature during aeration 16°–17° C.

the tenth hour. These observations were made at 17° C. In fig. 4, A and B, will be found curves showing the same phenomena as they occur at 37° C.

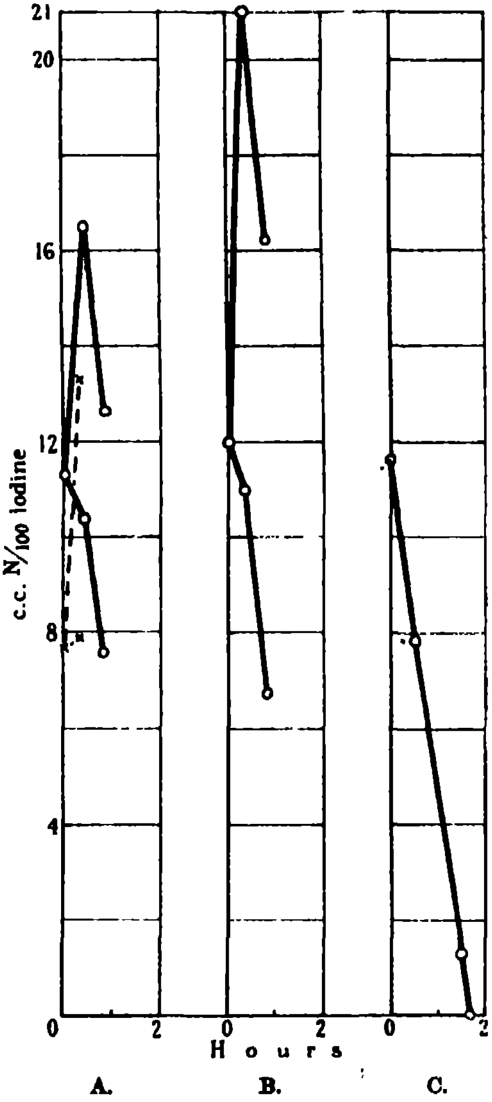


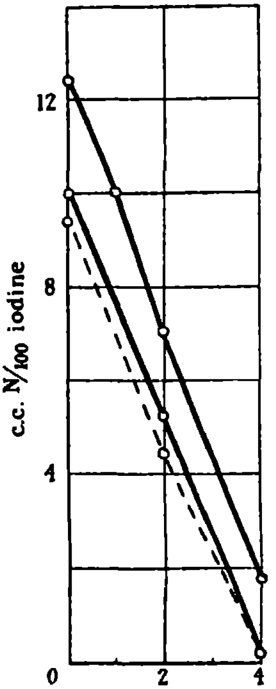
Fig. 4, A and B. Curves similar to those of fig. 3A but from experiments at 37° C. They show the result of adding GSSG before aeration. Fig. 4C shows the rapid disappearance of —SH from liver of fasting rabbit aerated at 37° C. after preliminary heating to 50° C.

The effect of inactivating the reducing system or systems in the liver should next be noted. This is accomplished by heating the chopped tissue to 50° C.

before aeration. The result is illustrated by the curves of fig. 3B. The upper pair with continuous lines are from the same liver. The uppermost is the normal curve, the animal being well fed. The companion curve was given when each of the samples titrated had been heated to 50° C. for 1 hour before aeration. The dotted lines show the results of a precisely similar experiment. The lowest curve in the same figure again shows the oxidative fall in GSH concentration in the case of another liver heated to 50° C. for an hour; no control being in this case carried out. Yet another case of the observed course of oxidation in a liver so heated is given in the upper curve of fig. 5. All these results were obtained at room temperatures; fig. 4C shows the same phenomena at 37° C. That the reducing systems are greatly reduced in activity by heating at the temperature mentioned is shown by the two lower curves of fig. 5. Of these the continuous line gives the results from a heated liver; the lower, dotted line those from the same liver when GSSG had been added before aeration. No rise in GSH concentration like that in the experiments of figs. 3A and 4 is here displayed, and no apparent delay in oxidation during the first 2 hours. It might be suggested that the effect of heating is due, not to the destruction of thermolabile catalysts, but to the exhaustion of the actual processes of reduction owing to their acceleration at the high temperature. Since in the excised tissue the GSSG is from the outset fully reduced, this could only be the case if reduction were accompanied by oxidation during the heating. We have found, however, that the result is precisely the same when the tissue is heated at 50° C. in fully evacuated vessels.

It is noteworthy that the rate of oxidation of GSH in livers after heating to 50° has in all experiments proved to be linear throughout. The explanation of this cannot, in the circumstances of our experiments, be ascribed to the oxygen supply as a limiting factor in the velocity of oxidation. It would seem rather that a catalytic agency which determines the oxidation of —SH remains saturated throughout. This cannot be the oxidase-cytochrome system of Keilin, or Warburg's "Respiratory Enzyme," as the presence of a high concentration of CO is quite without effect upon the rate of oxidation. It cannot indeed be enzymic in nature as it remains intact when the tissue is heated to 70° C. for an hour. The system is cyanide sensitive, however, and the catalyst, to judge from Meldrum and Dixon's observations, may well be iron in combination with cystine. The amount of the latter necessary to supply sufficiency of an active compound of the kind would, of course, be small; much less than could be detected by any colour test.

An experiment in which a substance (2 amino-phenol-4-sulphonic acid), claimed by Eichholtz (1929) and others to be an inhibitor of iron catalysis, was added to the tissue in a concentration of 1 in 250 showed no inhibition. Pyrophosphate M/30, however, undoubtedly inhibits, and so does α α dipyridyl.



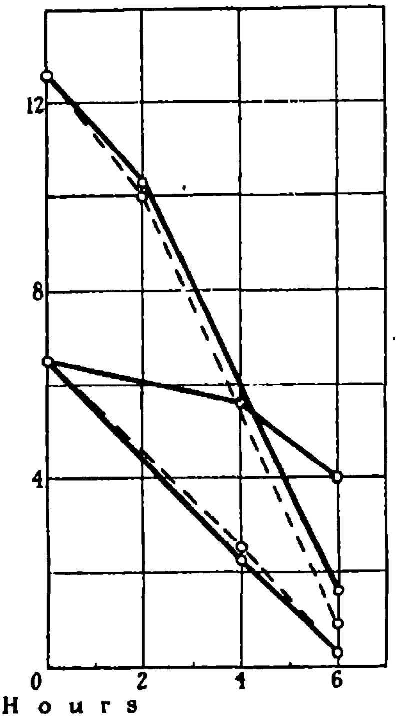


Fig. 5.—Showing absence of rise in —SH concentration on adding GSSG to well-fed rabbit's liver previously heated to 50° C. Lower continuous line shows normal linear fall in —SH ; dotted line applies to same heated liver after addition of GSSG. The upper continuous line again shows linear fall in the heated liver of another rabbit. Temperature 17° C.

Fig. 6.—Effects of CO and KCN. *Upper two curves*, continuous line displays normal disappearance of —SH ; dotted line, disappearance of —SH from same rabbit liver in presence of 78 per cent. CO and 10 per cent. O_2. *Lower three curves.* The lower pair again show disappearance of —SH in presence and absence of CO. The upper line shows the inhibitory effect of M/400 KCN. All three curves from the same rabbit's liver (fasting). Temperature 17° C.

In fig. 6 the two upper curves show respectively the course of oxidation in air (continuous line) and in a mixture containing 78 per cent. CO and 10 per cent. O_2 (dotted curve). The effect of CO is clearly negligible. In the same figure the three lower curves show results obtained from the liver of another animal. The lower pair show as before the absence of inhibition by CO, while the uppermost of the three show the effect of adding M/400 KCN. Such inhibition by the latter might be supposed to lack significance since, as is well known, cyanides reduce the disulphide form in alkaline solution. At p_H 7·6, however, and with the above concentration of cyanide, the reduction is very slow, as special experiments have shown. It was probably without effect upon the curve in fig. 6.

Livers of Oxen and Sheep.—Behaviour in these is essentially the same as in the livers of rabbits, except that the processes are characteristically slower. We were unable, however, to control the food intake of these animals. Their livers were obtained from the slaughterhouse and any information obtainable as to the hours of previous feeding was always vague.

Fig. 7 refers to two ox livers studied at 20° C. Each pair of curves shows the observed rate of oxidation in one of these; the lower curve with no addition made, the upper after adding GSSG. To judge from the experience with

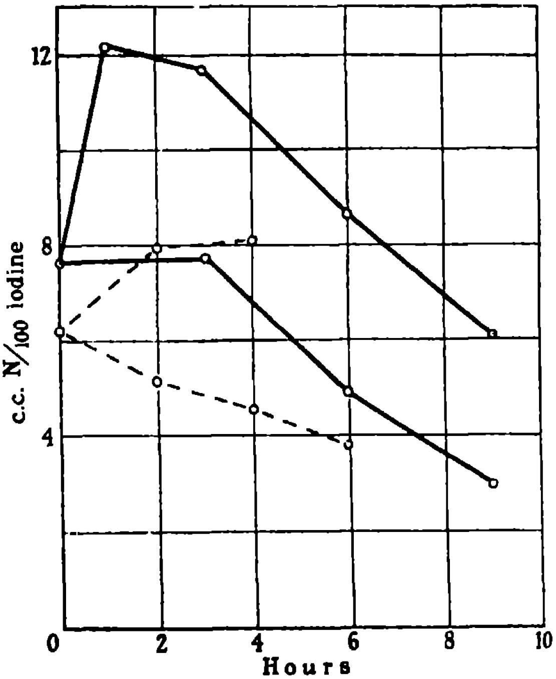


Fig. 7.—*Ox Livers.* Reduction of added —SS— and disappearance of —SH on aeration. The continuous lines refer to the liver of one individual, the broken lines to that of another. The upper curves in each case show the effect of adding 100 mgm. GSSG to each 10 gm. sample at the beginning of aeration. Temperature 20° C.

rabbits the upper pair refers to an animal recently fed and the lower pair to one kept longer without food. Fig. 8 depicts results from another ox liver in similar circumstances but at 37° C.

Fig. 9 refers to a sheep's liver, again with like treatment; temperature 20° C.

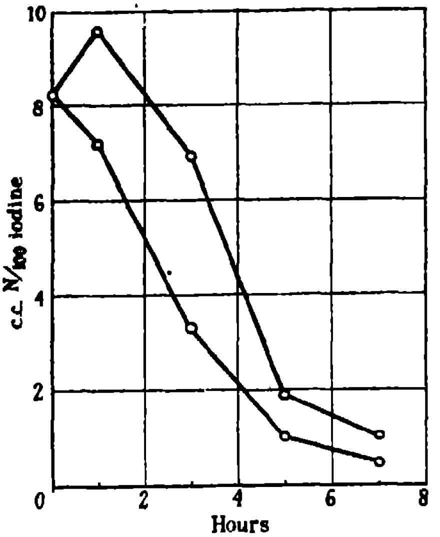


Fig. 8.—*Ox Liver*. Reduction of added —SS— and disappearance of —SH on aeration. The upper curve shows the effect of adding GSSG. Temperature 37° C.

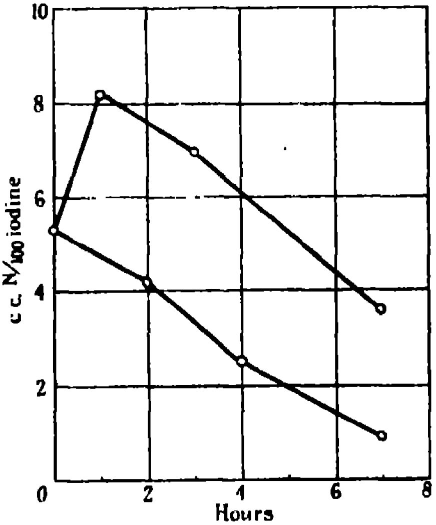


Fig. 9.—*Sheeps' Livers*. Changes in concentration of —SH during aeration. Upper curve after addition of GSSG, lower curve without. Temperature 17° C.

Cat Livers.—For some reason the liver of the cat shows a much higher capacity for reducing GSSG than that of any other animal studied. This sometimes appears in the low velocity of the observed rate of oxidation in livers aerated without any addition. The three curves of fig. 10 were obtained from such livers. The upper curve was from a well-fed animal, the two lower from animals that had received no food (but were given water) for 48 hours before the experiment. It will be seen that the apparent rate of oxidation was relatively very slow. The control curves in figs. 11 and 12B show, however, a higher initial velocity, but variations in the metabolic condition of the liver may well be expected.

The high reducing capacity of the cat's liver is shown consistently in a striking way when GSSG is added to the tissue; the result being illustrated in figs. 11 and 12. In the two latter it will be seen that the rise in the concentration of GSH as determined at the end of the second hour was too great

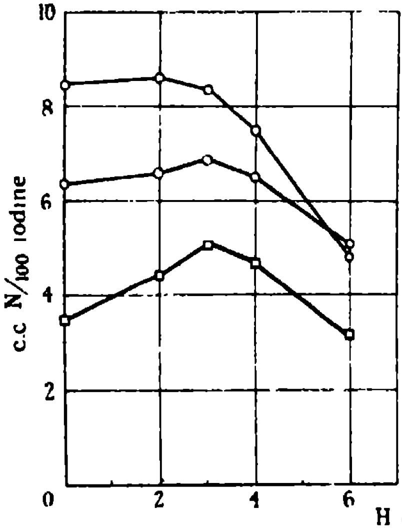


FIG. 10.—*Cats' Livers.* Upper curve from liver of well-fed animal. Two lower curves from livers of fasting animals. Temperature 17° C.

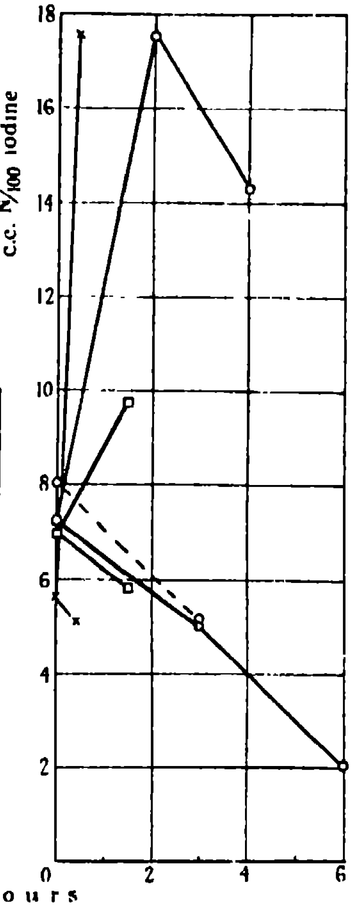


FIG. 11.—*Livers of well-fed Cats.* Significance of curves as in other figures.

— o — o — changes in —SH concentration with (upper curve), and without (lower curve) added GSSG.

— — — o — — — o — — — fall in —SH in same liver aerated after heating to 60° C.

— □ — □ — from liver of second cat at 17° C.

— × — × — from liver of third cat at 37° C.

to be demonstrated on the figure without abbreviation. This large rise occurred in spite of the vigorous aeration used in the experiments.

But the cat liver shows a remarkable contrast with those of the other animals

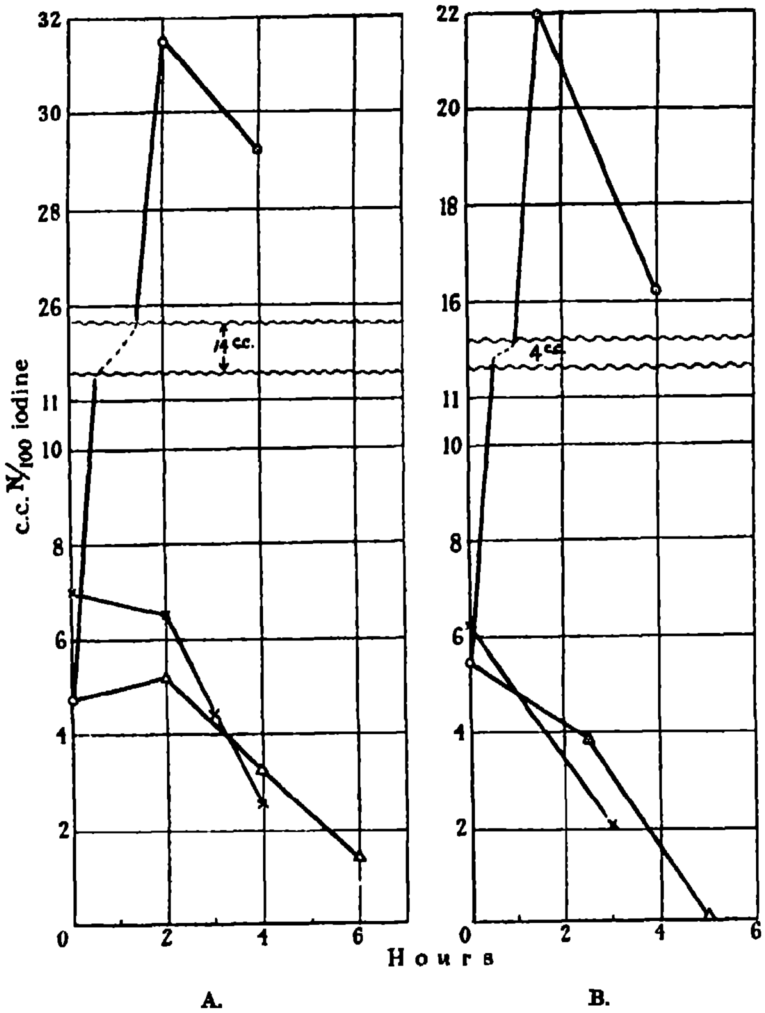


Fig. 12.—*Livers of Fasting Cats.*—A and B. Identical experiments on two individuals. Temperature 17° C.

— Δ — Δ — normal course of change in —SH during aeration.

— × — × — after previous heating to 50° C.

— o — o — after adding 100 mgm. GSSG to each 10 gm. sample. Note very large rise of —SH during vigorous aeration.

studied in that the greatest reducing power was displayed in the case of animals deprived of food instead of those fully fed (see legends of figures). These facts are discussed in the next section.

Dog Livers.—The high reducing power of the cat liver does not seem to be an essential characteristic of the organ in carnivora. It does not appear in the liver of the dog. We have, it is true, studied only two instances of the latter, and in neither animal was the intake of food controlled. The curves of fig. 13 show, however, that though the addition of GSSG (upper curve) produced a definite increase of GSH during aeration it was relatively small. It is noteworthy, however, that the observed rate of oxidation without added disulphide showed no delay during the first 2 hours, so the animals may have been for some time without food.

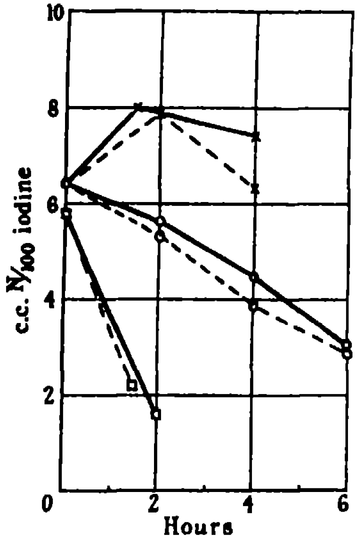


Fig. 13.—*Livers of Dogs.* Continuous lines refer to one individual, broken lines to another. The middle pair of curves show normal changes in —SH concentration ; the upper the effect of adding GSSG and the lowest pair the effect of previous heating to 50° C. Temperature 17° C.

Rat Livers.—A few observations were made upon these. The results were in marked contrast with those given by the livers of the larger animals. The velocity of the processes which reduce GSSG appears to be exceptionally small in the rat liver, as was earlier indicated by experiments described in section 2. On the other hand, the oxidative mechanisms seem to be particularly active in rat livers. There is no delay in the observed rate of oxidation during the earlier period of aeration and no difference is produced by 30 hours of food deprivation (fig. 14A), while the addition of the disulphide produced only a very small falling off in the apparent rate of oxidation during the first hour (fig. 14B).

Although the experimental results described refer to hepatic tissue only it is probable that, in essentials, they will hold for other tissues. We have, for

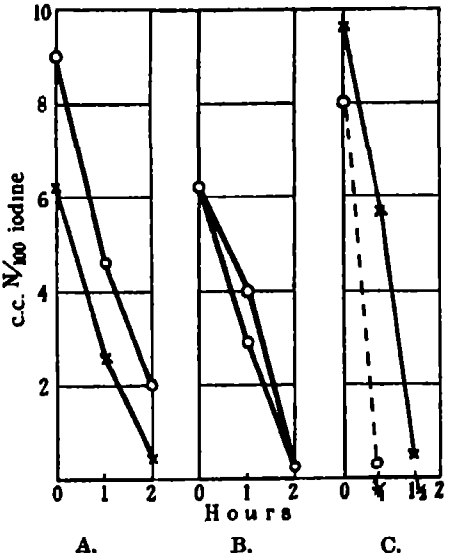


FIG. 14.—*Rat Livers.* A, Normal fall in concentration of —SH; upper curve in livers of well-fed animals, lower curve in liver of animals after fasting for 30 hours. B shows the relatively slight effect of adding oxidised glutathione before aeration (upper curve). C, continuous line, normal fall in mixed livers of well-fed rats; broken line, rate of fall in same mixed tissue after previous heating to 50° C. Temperature 17° C.

instance, made a few preliminary experiments with renal tissue and the results justify this statement.

Further Note on the Titration Method. Now that our experimental results have been presented it may be well that something should be added to what was said in the last section concerning the adequacy of the titration method as employed by us. Tunnicliffe's method is often suspected of giving results that are too high. Kühnau, however (*loc. cit.*) after investigating the matter with much care, has shown that no known metabolite likely to be present in trichloracetic acid extracts from the liver exerts such power of iodine absorption as to affect the end point appreciably when GSH is titrated. He supplies further evidence to suggest that any such interference by unknown metabolites is unlikely.

It is possible that series of titrations may give "uniformly" slightly high figures. Our experience shows this to be unlikely, but in any case the actual form of the curves will be unaffected. It is evident that when, after adding the oxidised form of glutathione to the liver tissue, a large rise in the iodine titration figure is observed during the early period of aeration, this can *only* be due to an increase in SH; reduction then temporarily outbalancing oxidation. However great this rise the titration figure during continued aeration ultimately falls to zero. When then in other circumstances we find that the titration figures during the earlier hours of aeration may show either an increase, no change, or a relatively slow decrease; while, later, the fall is in all cases more rapid and continuous; it cannot be doubted that the apparent delay in oxidation so indicated is due to the continuance of normal reduction processes of greater or less activity, which, later, in excised tissue, become exhausted.

§ IV.—DISCUSSION.

The experimental results described in the foregoing section show that the reduced form of glutathione, when present with its various normal associations in hepatic tissue, is oxidised to the disulphide form by molecular oxygen, the titratable —SH group gradually disappearing during the survival respiration of the excised tissue. From the livers of rabbits, for example, it may disappear as the result of 6 to 8 hours' aeration at room temperature, the time varying, of course, with the original concentration, while at 37° C. it may become wholly oxidised in 3 hours or less.

Such apparent rates, however, are resultant rates due to associated reduction and oxidation. Processes which reduce the SS group of the oxidised form continue during survival respiration, their velocity depending primarily upon the existing concentration, or the rate of activation, of certain hydrogen donators in the liver cells. The velocity of the oxidation of the reduced form as observed has, therefore, a resultant value depending upon the progress of two opposed reactions.

The existence of these relations is shown by the following evidence. First, by the circumstance that when the liver tissue is thoroughly aerated the concentration of GSH does not necessarily fall at all during the earlier period of survival respiration. It may remain constant (at room temperature) for 2 hours or more, and only then display the fall due to oxidation. This behaviour was shown consistently, for instance, in our experiments on the livers of well-fed rabbits (fig. 1). In other cases there may be an actual rise in the concentration during the earlier stages of aeration, though it is always followed by a fall and, ultimately, by the disappearance of the titratable —SH group. That in the first case oxidation is temporarily balanced by reduction, while in the other cases, in spite of an ample oxygen supply, the latter predominates, is shown by the circumstance that in all cases if the liver tissue be heated to 50° C., a treatment which greatly lessens its capacity to reduce GSSG (fig. 5), then from such heated tissue the GSH disappears during aeration with a velocity which is linear from first to last (figs. 3A, 4C and 5). Moreover, at a late stage in the survival respiration of the unheated tissue, when the concentration of GSH has fallen to a fraction of its initial value, if the conditions are then made anaerobic, a return rise in its concentration follows, showing that reduction processes continue, though with falling velocity, throughout the course of the oxidative disappearance.

That the velocity of reduction is greatly affected by the concentration of metabolites capable of acting as hydrogen-donators is indicated by the result of varying the nutritional condition of the animals. While, for instance, in the liver of a well-fed rabbit reduction and oxidation are so balanced during the early stages of survival that the concentration of GSH remains nearly constant, in that of a rabbit which has been deprived of food for 48 hours before the experiment oxidation is from the first more rapid than reduction and the concentration falls sharply in the earliest stages of aeration as well as in the later (figs. 1 and 2).

The observed rate of oxidation may vary in livers from different species of animal, even, it would seem, when the nutritional conditions are the same. Of the animals employed, however, full control of the diet was only secured in the case of rabbits and cats. In the former, as already stated, relatively brief deprivation of food leads always to a decrease in reduction processes in the liver, and, therefore, to an apparent increase in the rate of oxidation. This, we have reason to believe, occurs in most other species, but the cat would seem to form a striking exception; a circumstance which will be presently discussed. Whatever the nutritive condition it is probably true that the oxidation of GSH in the livers of oxen, sheep and dogs (figs. 7, 9, 13) is slower than in that of rabbits.

In any endeavour to appraise the significance of the respiratory phenomena in question a fundamental circumstance should be borne in mind. The pressure head—so to speak—of active hydrogen, capable of reducing the SS group of oxidised glutathione, may be quantitatively important. It is, as we have seen, an influence which always limits the observed velocity of —SH oxidation, but its magnitude in certain conditions is fully demonstrated only when the concentration of glutathione in the tissue is increased by adding an excess of the oxidised form (figs. 3A, 7, 11, *et al.*). When, after this addition, the progress of survival respiration is followed it is found that, in spite of brisk aeration of the tissue, there may be, during the first hour or two of survival, a large rise in the concentration of GSH. The velocity of reduction then notably exceeds that of oxidation, though, ultimately, owing to the exhaustion of reducing factors in the excised tissue the whole of the reduced glutathione, that produced from the added oxidised form no less than that naturally present, is oxidised. The preliminary rise is far more marked in livers from well-fed rabbits than in those from rabbits deprived of food. In the former the concentration of GSH may show an increase of 70 per cent. when estimated at the end of the second hour of aeration (see figs. 3A, 4). For the same behaviour in the case of the ox liver see fig. 7, and for the dog liver see fig. 13. The rise after adding GSSG is exceptionally prominent in the case of the cat. In all circumstances indeed the liver of that animal displays high capacity for maintaining glutathione in a reduced condition. It will be seen from the curves of fig. 10 that the observed velocity of oxidation of GSH in cat livers, when no addition has been made, is commonly much slower than in the livers of rabbits. Since this is almost certainly due to the circumstance that reducing processes are more active, it is not surprising that the GSH concentration rises with exceptional rapidity after the addition of GSSG to the tissue. Remarkable, however, is the circumstance that, in the case of the cat, previous deprivation of food for 48 hours increases this activity in the liver instead of decreasing it as with rabbits. In one experiment with such a liver, in which excess of the disulphide form was added to the samples before aeration at room temperature, the concentration of the thiol form at the end of the second hour of aeration showed on titration a fourfold rise. In another precisely similar experiment it rose, in spite of the usual vigorous aeration of the tissue, to six times its initial value. In the liver from a fully-fed cat, on the other hand, it rose to 2⅓ times the initial value only. These results, as obtained at room temperature, are reproduced in figs. 11 and 12; fig. 11 shows the rise after 25 minutes at 37° C. As in all other cases, continued aeration results ultimately in complete oxidation of the thiol group. This noteworthy difference in the effect of food deprivation upon the systems which reduce the disulphide group in livers of cats and rabbits respectively is not easy to explain. It is possible that it is a difference between carnivora and herbivora, though we have not established this. We were not able to control food uptake in the case of oxen and sheep and we have not cared to work with dogs intentionally deprived of food, so that our experiments are not conclusive upon the point. In any case, however, the high reducing power shown by the cat's liver under all circumstances is not paralleled in the case of the dog's organ. It is possible to think that in the rabbit the metabolites, which serve as hydrogen donators in the reduction of GSSG, are directly derived from the gut and disappear in the absence of food, whereas in the cat they appear in greater concentration when the liver stores are mobilised as the result of food deprivation.

We have made relatively few observations on the livers of rats, but the results have been consistent. They are in sharp contrast with those obtained from the organs of larger animals and especially with those given by cat livers. Observations mentioned in section 3 (*a*) indicate that the reduction of GSSG in the rat liver is notably slow, a fact fully confirmed by the aerobic experiments. The apparent rate of oxidation is exceptionally fast throughout, and shows little or no delay during the earlier periods of aeration; while addition of GSSG results in but a very small rise in the iodine titration figures (figs. 14A and 14B).

These facts may perhaps point to some general character in the hepatic metabolism of these small animals of which the behaviour of glutathione is an indicator.

Nevertheless processes which reduce glutathione are not absent from the liver of the rat. This is shown in the anaerobic experiments, and by the circumstance that when the tissue has been heated to 50° C. the oxidation of GSH is notably accelerated (fig. 14c). Hydrogen transport to oxygen undoubtedly occurs, but it would seem that during life the dynamic equilibrium SH $\rightleftarrows$ SS may be maintained in the rat at a level different from that which exists in the livers of the other animals studied.

The facts which have been discussed yield evidence, then, that while the oxidation of reduced glutathione is, in the hepatic tissue, a continuous process, its concentration may nevertheless be maintained by the continuance of reduction processes. In the case of the excised tissue this is shown plainly enough in the early periods after excision, while at later stages of survival respiration the progress of oxidation is uncovered by the partial exhaustion of reducing factors. It is probable that while the organ is *in situ*, with an intact circulation, the concentration of hydrogen donators, and therefore the concentration of the thiol group, is maintained at some average level. In any case it would appear certain that hydrogen in no negligible amount is continuously transported by this path from tissue constituents to molecular oxygen.

We may endeavour to decide what oxygen uptake is represented by such transport. If, as the absence of inhibition by CO (fig. 6) indicates, the availability of oxygen for this system does not depend upon the functions of the oxidase-cytochrome system or of the "Atmungsferment," the proportionate uptake, to judge from the classical work of Keilin and Warburg, must be expected to be relatively small.

It is legitimate to suppose that the real rate of oxidation of GSH in the tissue is that displayed when it is not cloaked by reduction. In fig. 4 (c) is shown the linear fall in its concentration, observed at 37° C., in the liver of a rabbit previously deprived of food for 48 hours, the tissue having been first heated to 50° C. Reduction processes being thus nearly eliminated, the velocity of oxidation was near its maximum for excised tissue. The GSH present (34·8 mgm. in 10 gm. of tissue) was completely oxidised in 1¾ hours. This will be found on calculation to correspond to an uptake of 41 cmm. of oxygen per gm. of tissue per hour. This figure is at best only provisional, and since the rate of respiration in surviving mammalian livers is still under discussion (see Dixon and Elliott, 1929, and Warburg *passim*) it would be premature to decide what actual proportion of the whole it represents. It is doubtless a small fraction.

The actual significance of such a relatively small fraction of metabolism cannot, however, be appraised until the nature of the whole system involved in its progress, and its relation to other systems, are known. At present the nature, alike of the hydrogen donators concerned in the reduction of glutathione, and of the mechanisms which activate them, is uncertain. The latter are partly destroyed at 50° C., and this Keilin has shown to be true of dehydrogenases in yeast. One of us (Elliott, 1928) has found, however, that the succinic acid dehydrogenase and the purine dehydrogenase in the presence of their substrates are both incapable of reducing GSSG, and the same is true of other dehydrogenases since tested. An adequate reduction potential does not seem to be established by any of these known systems. So far, moreover, no known metabolite has been found which when added to liver tissue increases the rate of reduction.

Some time ago one of us (Hopkins, 1925) showed that proteins which display a " fixed SH group " reduce GSSG and suffer some form of oxidation under its influence. This observation has been often confirmed since, but as a phenomenon it can play but a small share in the hydrogen transport that we are considering. It cannot, for instance, form part of the reducing mechanisms which are destroyed at 50° C.

Meyerhof's experiments (1923) have shown that the --SH group catalyses the oxidation of fats, but, according to that author, as a species of oxygen activator rather than as an agent for hydrogen transport. This circumstance indirectly adds importance to the system with which we are concerned, but if the facts have been rightly interpreted it has, of course, nothing to do with the reducing systems of which the nature is in question. The same applies to the production of hydrogen peroxide during the oxidation of SH, which, as Thurlow (1925) has shown, may, in the presence of peroxidase, bring about secondary oxidations.

We do not know then the source of the hydrogen which reduces GSSG, or, in other words, the tissue constituents which suffer oxidation by the particular process of hydrogen transport with which this paper is concerned. If they or the mechanisms which activate them should prove to be specific the interest of this aspect of tissue respiration will increase, particularly as in respect of oxygen activation it is also specialised, being apparently independent of the oxidase cytochrome system or Warburg's Respiratory Enzyme. An endeavour to define the hydrogen donators is in progress.

A very noteworthy circumstance is the contrast between the behaviour of glutathione in the survival respiration of mammalian tissues, as shown by our experiments, and the great apparent stability of the thiol form in the yeast cell as demonstrated by Meldrum (1930).

Meldrum's observations suggest, though they do not perhaps conclusively prove, that the constancy of the SH concentration in respiring yeast is not due to its maintenance by reducing processes. If it be independent of these the function of glutathione in animal tissues and yeast respectively must be different. The question certainly deserves further study.

There is one consideration to which no attention has yet been given. Bierich and Kalle (1928) and others have shown that glutathione undergoes hydrolysis in the excised liver. The question therefore arises as to whether in the experiments described oxidation of the thiol group may not have occurred, at any rate in the later stages of the process, in free cysteine rather than in the intact tripeptide. There is no doubt that adequate aeration and controlled p_H much delay the onset of autolytic processes. In view of the above possibility we nevertheless endeavoured to estimate by Sullivan's method the free cystine present towards the end of oxidation. For some reason the method in our hands proved unsatisfactory from a quantitative standpoint when applied to liver extracts. We finally came to the conclusion that a small amount of cystine, equal to some 10 per cent. of that in the tripeptide, may be free at the end of the fifth hour at room temperature, though it does not follow that all is derived from glutathione. By that time, however, the greater part of the oxidation of the SH group has been completed.

SUMMARY.

The relative capacity of various mammalian tissues to reduce the disulphide form of glutathione under anaerobic conditions has been determined.

The course of the oxidation of the thiol group during the survival respiration of the hepatic tissue of various animals has been followed.

During the earlier periods of aeration after excision, the fall in the concentration of —SH in the livers of well-fed animals is slow. It may indeed remain constant for some time, or even rise. Such delay is, however, always followed by a progressive fall, and, ultimately, by the complete oxidation of the thiol group in the tissue.

It is shown that the preliminary delay is due to the continuance during survival respiration of reducing processes in the tissue. The rate of disappearance of —SH, as observed, is the resultant of associated reduction and oxidation. The agencies responsible for the former become gradually exhausted in excised tissue, and the observed velocity of oxidation then increases.

That the concentration of certain metabolites in the liver is wholly, or in part, responsible for the maintenance and amount of reduction is shown by the effects of administering or withholding food. In general the initial marked delay in the disappearance of the – SH group during aeration is observed only in the liver of well-fed animals; after relatively brief periods of fasting it is much lessened and may not be observed.

That thermolabile catalysts are concerned in the reducing processes is shown by the circumstance that if the liver tissue be heated to 50° C., under anaerobic conditions, before it is aerated, the observed disappearance of —SH is much more rapid. It is then continuous from the first and follows a linear course.

If extra glutathione in the disulphide form be added to the freshly excised tissue before aeration, the existence of residual active hydrogen is revealed. In spite of vigorous aeration the concentration of —SH then rises during the earlier periods of survival, though ultimately the whole is oxidised. The amount of this extra pressure-head of hydrogen consistently differs in the livers of different species. In that of the cat the concentration of —SH may reach six times its initial value before the fall which leads to its ultimate disappearance begins. In those of the ox, sheep, rabbit and dog the rise is marked but less considerable. In the liver of the rat, in which the oxidation of —SH is exceptionally rapid, it is scarcely to be seen.

The origin of the active hydrogen which reduces the disulphide grouping is at present unknown. Carbon monoxide does not inhibit the oxidation of the thiol group, which is therefore not due to the influence of iron-hæmatin compounds. It is almost certainly catalysed by iron in some association however, being inhibited by cyanides and pyrophosphates. The oxidation is not enzymic as it proceeds normally after the tissue has been heated to 70° C.

The transport of hydrogen to molecular oxygen by the path in question, though representing but a small part of the total respiration, may therefore prove to represent a specialised and significant aspect of tissue oxidations.

Three of the curves reproduced in the paper were obtained independently by W. J. Dann. A great part of the manipulative work involved in the experiments was carried out by E. J. Morgan.

The expenses of the research were entirely covered by grants to one of us (F.G.H.) from the Medical Research Council.

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Interfacial Tension and Hydrogen-ion Concentration.

By Rudolph Albert Peters, from the Department of Biochemistry, Oxford.

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Abstract.

(1) Long chain carboxylic acids dissolved in benzene show regular changes in interfacial tension against aqueous "buffered" solutions as the hydrion concentration of these is altered. A fall in interfacial tension starts at p_H 5·5 and extends over the range of 4·0 p_H units to p_H 9·3 approximately, tending to vanish at this point. The curve is not identical with a dissociation curve, though it extends over the same range of p_H. For a given p_H the results are identical for phosphate and glycine "buffered" solutions, and for all acids investigated, except capric acid (C_{10}), which shows an abnormality for phosphate.

(2) Hexadecylamine shows similar changes, in the opposite sense between

approximately the same p_H range, which follow the dissociation curve of a weak base rather closely.

(3) The K_a and K_b of —COOH and —NH_2 in the interface can be calculated approximately from these results to be $3 \cdot 2 \times 10^{-8}$ and $1 \cdot 0 \times 10^{-7}$.

(4) Ethyl palmitate shows a fall of similar type to that for the carboxylic acids, but starting at a more alkaline point. Hexadecyl alcohol exhibits no change.

Some Biological Considerations.

The biological application of these results deserves some comment. The effects studied here occur over the range of p_H which is the mean reaction of the living cell p_H 7·3. At this reaction, simple acids and bases in true aqueous solution would be completely present as salts. At the interface this should not be so, and therefore these groups might take part in dynamic actions of the cell.

It is surely significant that the curves for amine and carboxylic acid cover approximately the same p_H. A problem of some interest in living matter is raised by the presence of fatty substances in interfaces and their probable exposure to varying H-ion concentrations. Why do they not tear the cell to pieces ? It is likely that the living cell is buffered against changes in interfacial tension in its various parts. Such buffering must readily take place with a mixture of amine and an acid such as the above. A few experiments with a mixture of amine and carboxylic acid in benzene indeed indicate that this is so ; such a mixture showed the following I.T. at various p_H :—

p_H	5·4	6·3	8·0	10·42
I.T.	9·40	13·8	19·7	6·15

At either extreme of reaction there was definite evidence of "tension" buffer effect.

The view that muscular motion might be produced by changes in surface tension (Bernstein) has been variously treated, receiving support from Hartridge and Peters (*loc. cit.*), and adverse criticism from Harkins, Davies and Clark,* and A. V. Hill.† Hartridge and Peters (*loc. cit.*) pointed out that if COOH groups formed part of the protein structure of the contractile mechanism of a muscle, their work indicated the way in which changes of interfacial tension might be caused by changes in C_H. Later somewhat similar views have been advanced by Garner[*] and Meyer.[†] Definite evidence has been here produced that $-NH_2$ as well as $-COOH$ groups can take part in such effects. The groundwork for the theory that protein structure may react to experimental variations in p_H by mechanical changes therefore exists, though recent work (Meyerhof) suggests that muscular contraction may not be associated with changes in p_H.

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* 'J. Amer. Chem. Soc.,' vol. 39, p. 354 (1917).

† 'Proc. Roy. Soc.,' B, vol. 98, p. 506 (1925).

It is well known that certain cells of the central nervous system (the so-called respiratory centre) are sensitive to changes of p_H of the order of 0·02, and probably rather less. At a p_H 7·4, 0·01 p_H corresponds to a change of about 0·14 dyne for the amine and rather less for the acids. It is quite possible that this is sufficient to initiate the appropriate response.

(*The full paper is printed in* 'Proceedings,' A, *vol.* 133, *pp.* 140–154.)

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[*] 'Proc. Roy. Soc.,' B, vol. 99, p. 40 (1925).

[†] 'Biochem. Z.,' vol. 214, p. 253, and vol. 217, p. 433 (1929).

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Studies on the Flexor Reflex. VI.—*Inhibition.*

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(Received August 21, 1931.)

[Plates 8, 9.]

I. Introduction.

Since it was first described (Sherrington, 1907; 1909; Sherrington and Sowton, 1911), inhibition of the flexor reflex by stimulation of contralateral afferent nerves has not been so systematically studied as inhibition of extensor reflexes. This comparative neglect is possibly due to its frequent absence when sought for against the background of a tetanic flexor reflex (Graham Brown, 1914). Sherrington and Sowton (1915), however, showed that a series of flexor reflexes evoked by single break-shocks could be regularly inhibited from contralateral nerves, and by using this technique it was possible to demonstrate the slow passing off of inhibitory after-effect subsequent to the stimulation of a contralateral nerve.

An important contribution to the study of inhibition was published by Samojloff and Kisseleff (1927), who employed single flexor reflexes or slow series of single flexor reflexes as backgrounds for the investigation of the inhibition arising from the stimulation of contralateral nerves. Their most important finding was that the flexor reflex evoked by a single excitatory volley suffered inhibition from a single inhibitory volley (contralateral) which preceded the excitatory volley by intervals between 10 σ and 300 σ. The present investigation is an attempt to study the nature of this long-lasting inhibition.

II. Method.

The experimental technique and apparatus have been identical with those described in the previous series of papers on the flexor reflex (Eccles and Sherrington, 1931, *a*). In all experiments the preparation has been decerebrate with the spinal cord cut through at the 12th thoracic segment, and in most the flexor muscle (tibialis anticus in all experiments, except a few with semitendinosus) has been completely deafferented. Complete deafferentation has been the rule in all experiments where an antidromic volley has been set up. Usually a break-shock applied to the ipselateral popliteal nerve has been employed to evoke the flexor reflex and this has been inhibited by a single break-shock to the contralateral peroneal nerve. In all experiments except those involving a tetanic inhibition a Lucas pendulum has been used to set up the stimuli at known intervals apart.

The time between the dissection and the experimental investigation has been sufficiently long for the discharge of impulses from the cut nerve ends to cease (Adrian, 1930). Transection of the spinal cord and deafferentation of the flexor muscle ensure that its motoneurones are as nearly as possible in a resting condition when stimuli are not being applied to the afferent nerves. Successive observations have been taken at intervals usually of 1 minute in order to allow the spinal cord to attain a resting condition before each period of inhibition. All experiments deal, therefore, with the inhibition of resting motoneurones.

III. The Reflex Pathways.

Fig. 1 is a diagram of the simplest type of fibre connections involved in the contralateral inhibition of the flexor reflex. Stimulation applied by the electrodes E sets up centripetal impulses in the excitatory afferent, and these impulses may be incident directly on the flexor motoneurones (Eccles, 1931). The impulses set up in the inhibitory afferent by electrodes I are, however, not directly incident on the contralateral flexor motoneurones, for histological study fails to reveal the passage of any afferent nerve fibres to the contralateral anterior horn (Cajal, 1909). There is at least one internuncial neurone in the inhibitory pathway. Electrodes A on the intact motor nerve are used

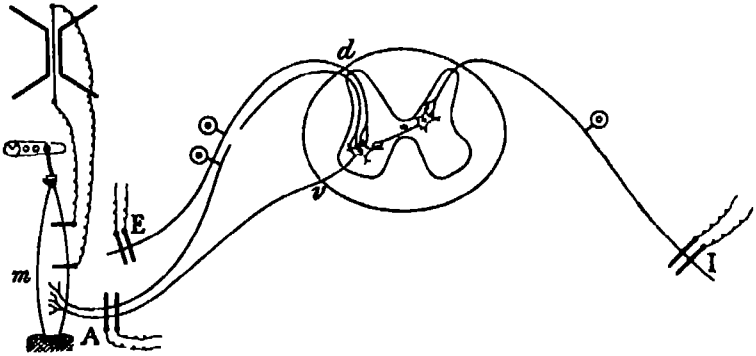

Fig. 1.—Diagram of the simplest type of fibre connections for contralateral inhibition of the ipselateral flexor reflex.

for setting up antidromic impulses, which pass up the motor nerve fibres to the motoneurones.

For convenience throughout this paper the volley of impulses set up in the inhibitory afferent nerve by a stimulus applied through electrodes I has been called an inhibitory volley. The impulses of this volley are, of course, ordinary nerve impulses having no relation to inhibition. At the terminal endings of the fibres along which they travel, these impulses give rise to c.e.s. which may set up discharges in internuncial neurones. The impulses set up from these or other internuncial neurones further on in the series, when incident on motoneurones, may give rise to inhibition (Sherrington, 1906, p. 192; 1913; 1925). This inhibition is not a conducted state. So far as is known the impulses which give rise to inhibition do not in themselves differ from those which give rise to excitation. The differences arise at the terminals.

IV. EXPERIMENTAL INVESTIGATIONS.

1. *The Inhibition of a Reflex Twitch by a Single Inhibitory Volley.*

As has been shown by Samojloff and Kisseleff (1927), the flexor reflex evoked by a centripetal volley in an ipselateral nerve is inhibited by a centripetal volley in a contralateral nerve when this latter volley is set up at certain intervals before the excitatory volley. It has been found that the amount of the inhibition produced by a given inhibitory volley varies with the size of the reflex against which it is pitted, the interval between the excitatory and inhibitory volleys being, of course, kept constant.

Table I.

Coil distance for the stimulus applied to the excitatory nerve.	Tension of reflex alone, in grm.	Tension of inhibited reflex, in grm.	Amount of inhibition, in grm.
cm. 27	364 334		
	349 average	138	211
16	1170 1126		
	1148 average	1032	116
27	287 299		
	293 average	103	190

In Table I measurements from such a series of observations are shown, one series at 27 cm. being taken before and the other after the series at 16 cm. The reflex alone was elicited before and after the inhibited response and the average tension is taken as shown in the table. The weaker reflex suffers a much greater inhibition than the stronger. Similar series of observations have been obtained in other experiments, and comparable experiments using tetanic excitations and inhibitions have been described previously (Sherrington, 1909, 1929). Since in the present series of experiments the reflex responses are reflex twitches, *i.e.*, there is no after-discharge (Eccles and Sherrington, 1931, *a*), any reduction in the tension of a reflex must be due to prevention of the reflex discharge of some motoneurones. If a statistical average is assumed for the tension of an individual motor unit (Eccles and Sherrington, 1930, *b*), the diminution in tension of the reflex twitch will be proportional to the number of inhibited motoneurones. Thus with the weak reflex in Table I the number of motoneurones inhibited is larger than with the strong reflex. Some of the motoneurones which respond in the weak reflex and then suffer inhibition must be included amongst those which, when taking part in the strong reflex, resist the inhibition. *It follows that in these motoneurones the prevention of reflex discharge by inhibition depends on the intensity of their excitation.* If this is powerful, the inhibitory process may be present but without effect--a condition comparable with the subliminal central excitatory state.

In some experiments, *e.g.*, Table II, however, the stronger the reflex the larger is the amount of inhibition produced by a given inhibitory volley. In such cases no conclusions can be drawn as to the effect which the degree of excitation of a motoneurone has on its resistance to inhibition.

Table II.

Order of observation.	Tension of reflex alone, in grm.	Tension of inhibited reflex, in grm.	Amount of inhibition, in grm.
4	29	8	21
9	68	13	55
7	118	26	92
5	189	87	102
3	201	67	134
8	224	88	136
1	290	144	146
6	352	163	189
2	466	227	239

In all experiments the amount of inhibition diminishes when the reflex becomes very small, there being fewer motoneurones to inhibit. Even with quite weak reflexes there are usually a few uninhibited motoneurones, *e.g.*, the observations of the first row of Table II. Such motoneurones are probably not included in the particular inhibitory field under investigation (Creed and Eccles, 1928).

If the excitatory volley be kept constant, the amount of the inhibition varies within limits with the strength of the stimulus setting up the inhibitory volley. However, with the exception of weak reflexes, the strongest stimuli fail to effect a complete inhibition.

2. *Time Relations of the Inhibition following a Single Inhibitory Volley.*

When the interval by which the inhibitory volley precedes the excitatory is varied, the amount of inhibition changes correspondingly (see Plate 8, fig. 1). The relationship in any particular series of observations is best expressed in graphical form. In fig. 2 the amounts of inhibition (measured by the reduction in the maximal tension of the reflex twitch) are plotted as ordinates against the corresponding intervals by which the inhibitory volley precedes the excitatory. The results of fig. 3 are derived from an experiment where the duration of the inhibitory process was much briefer. The observations from 14 other experiments give curves similar to those shown in figs. 2 and 3.

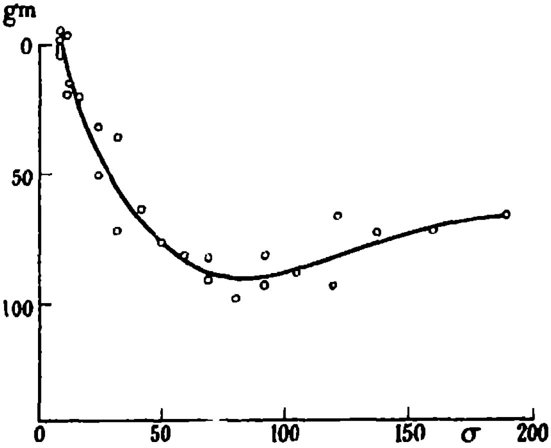

FIG. 2.—Amounts of inhibition in grams plotted as ordinates against the corresponding intervals by which the inhibitory volley precedes the excitatory (in sigmata). The base line shows the level of complete inhibition.

When the inhibitory volley precedes the excitatory by less than 6 σ to 8 σ there is never any sign of inhibition. The shortest intervals between the inhibitory and excitatory volleys at which inhibition is observed usually vary from 8 σ to 12 σ in different experiments, but the interval may be as long as 20 σ. Such long intervals occur in those cases where the contralateral centripetal volley exerts an early excitatory effect as well as an inhibitory effect on the flexor motoneurones, hence the delay in the apparent onset of inhibition.

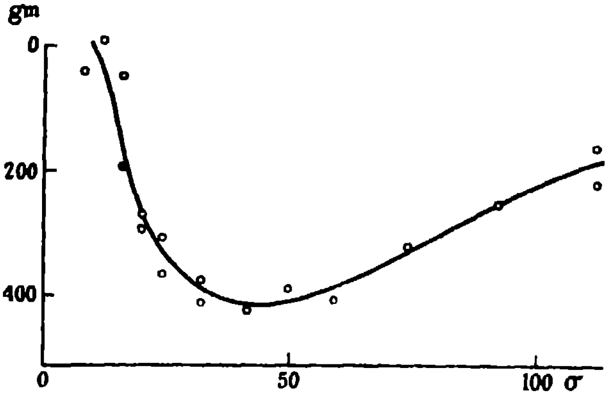

FIG. 3.—As in fig. 2 in another experiment.

Further increase in the interval between the inhibitory and excitatory volleys is accompanied by a progressive increase in the amount of the inhibition until a maximum is reached at an interval somewhere between 30 σ and 80 σ. At intervals beyond this there is a progressive subsidence of the inhibition. In most experiments the maximum stimulus-interval obtainable from the pendulum (200 σ) has not been sufficient to allow a determination of the longest interval at which inhibition could be detected. This interval has always been longer than 100 σ.

This simple type of curve with a single maximum has been obtained in all experiments; with the muscle deafferented or undeafferented (as was the case with the experiments of Samojloff and Kisseleff); with semitendinosus as well as tibialis anticus; and with inhibition from either peroneal or popliteal nerves. The size of the inhibitory volley has no appreciable effect on the time-relations of the resulting inhibition, *e.g.*, the latent period or the total duration, but, of course, it affects the depth of the inhibition at any particular time. As the ordinates can at best be considered proportional to the numbers of inhibited motoneurones, the intensity of the inhibition in the average motoneurone has no direct relationship to the reduction of the reflex response, but it seems safe to assume that the greater the number of inhibited motoneurones the more intense is the inhibition of the average motoneurone.

3. *Summation of Inhibition.*

(A) ***With Two Inhibitory Volleys.***—When two similar inhibitory volleys I_1 and I_2 in the *same* nerve are separated by a short interval, an ensuing reflex twitch suffers an inhibition greater than that produced by either volley alone (see Plate 8, fig. 2). Table III shows four such series of observations with different I_1 I_2 intervals. Since the distribution of the effect of the weaker volley must be completely overlapped by the stronger, this extra inhibition indicates that the two volleys are able to inhibit some motoneurones which apparently are unaffected by a single volley.

Table III.

1. Order of observation.	2. Stimulus-intervals, E signifies the excitatory volley.	3. Amount of inhibition by I_1 alone, in grm.	4. Amount of inhibition by I_2 alone, in grm.	5. Amount of inhibition by $I_1 + I_2$, in grm.
3	I_1 5 σ, I_2 33 σ E	347 (3)	379 (3)	423 (4)
1	I_1 8 σ, I_2 33 σ E	368 (2)	356 (2)	428 (2)
4	I_1 24 σ, I_2 33 σ E	355 (3)	333 (3)	425 (4)
2	I_1 41 σ, I_2 33 σ E	343 (2)	307 (3)	423 (4)

Note.—The figures in brackets in columns 3, 4 and 5 are the numbers of observations averaged to give the values shown.

It is conceivable that this effect could be due to facilitation between the inhibitory volleys when incident on the internuncial neurones which always exist in the inhibitory pathway (see fig. 1). Increased inhibition would result from the impulses so set up in additional internuncial neurones, but this inhibition should be much greater with short I_1 I_2 intervals, for only under such circumstances is facilitation likely to be appreciable (Eccles and Sherrington, 1930, *a*; 1931, *b*). But in all experiments the additional inhibition has shown but little change over a relatively wide range of I_1 I_2 intervals (*cf.* Table III). Therefore the alternative explanation on the basis of a summation of the inhibitory effects which each volley exerts on certain motoneurones seems much more likely to be correct. With excitation an analogous condition exists when two excitatory volleys at a short interval apart set up a reflex discharge from motoneurones which are in the subliminal fringe of either volley alone (Eccles and Sherrington, 1931, *b*).

Since inhibition can be summed it follows that different grades of inhibition are possible in a motoneurone (Sherrington, 1929, p. 355). This makes it likely that the additional inhibition of a given reflex by a larger inhibitory volley (see § 2) would also be due to an increase in the inhibition of some motoneurones from a latent to an effective intensity. In that case latent inhibition would be a true analogue to subliminal excitation.

The extra inhibition produced by the summation of the effects of two volleys is never very large and it is sometimes not easy to demonstrate at all. It is most obvious when each volley alone produces a similar amount of inhibition.

When the inhibitory volleys are set up in *different* afferent nerves, both volleys also inhibit more motoneurones than either one alone. This may be due to summation of inhibition, but it must be remembered that the same condition would arise if there were a failure of complete overlap between the inhibitory fields of each volley.

(B) *With a Repetitive Series of Inhibitory Volleys.*—Plate 8, fig. 3, shows part of a series of observations in which the condition of the flexor motoneurones is tested by an excitatory volley at various times during and after the repetitive stimulation of an inhibitory nerve. In fig. 4, the results of such a series are plotted with time as abscissæ and the amount of inhibition as ordinates. During the continuation of the inhibitory tetanus the intensity of the inhibition increases progressively, while shortly after its cessation the intensity of the inhibition decreases at such a rate that after 400 σ the flexor centre has almost

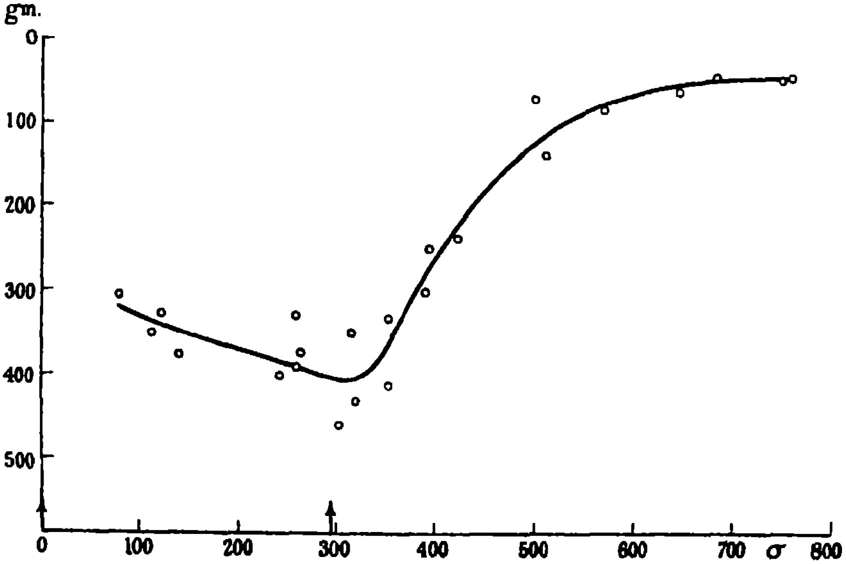

FIG. 4.—Amounts of inhibition of reflex twitches in grams plotted as ordinates against the corresponding time relations to a tetanic stimulation of an inhibitory nerve. The duration of this stimulation is shown by the arrows at the base line which represents the level of complete inhibition.

recovered its normal excitability. The comparative slowness of the increase in the intensity of the inhibition during the inhibitory tetanus parallels the relatively slight additional inhibition resulting from the compounding of two inhibitory volleys. The recovery of the normal excitability of the flexor centre following the repetitive inhibitory volleys is not noticeably different in rate from the recovery following a single inhibitory volley.

4. *The Effect of an Antidromic Volley on Inhibition.*

When an antidromic volley travels up motor nerve fibres to motoneurones, there is an inactivation of the central excitatory state of those motoneurones, and from this fact several conclusions have been drawn (Eccles, 1931 ; Eccles and Sherrington, 1931, *c*). The long duration of the inhibitory effect produced by a contralateral volley makes it possible to carry out similar experiments on inhibition.

Plate 9, fig. 4, shows a series of observations in which an antidromic volley, A, is set up at two different times between an inhibitory volley, I, and an excitatory volley, E. When the intervals are I 104 σ A, 14 σ E (observation 2), the small inhibited reflex action-current (*cf.* observation 5) follows the maximal motor action-current. This latter is, of course, a necessary result of setting up an antidromic volley (observation 4). With the intervals of this series the reflex response to E is not affected by the preceding antidromic volley, *e.g.*, the reflex action-current in observation 3 compared with those in observations 1 and 6. Therefore the inhibition by I of the reflex response to E has been unaffected by the antidromic volley. With the intervals of observations 7 and 8 (I 113 σ A, 5 σ E) the refractory period following A persists sufficiently long to diminish the reflex response to E (Eccles, 1931). The effect of the inhibition is, however, still present as evidenced by a further reduction in the response to E (*cf.* observation 7 with observation 8). The inferences drawn from the action-current records are confirmed by analysis of the myograms.

Many similar series of observations have been obtained in six other experiments and it has invariably been found that inhibition is unaltered by an antidromic volley. In observations 7 and 8, Plate 9, fig. 4, the antidromic volley reached the motoneurones at an interval shorter than 10 σ before their reflex discharge, and in some other series of observations this interval has been still shorter.

Table IV has been calculated from one such series, most of the values being averages of two or more observations. In rows 2 and 3 the values of column 7

Table IV.

1. Stimulus intervals.	2. Tension of uninhibited reflex alone = W grm.	3. Amount of inhibition of reflex by I = X grm.	4. $\frac{X}{W}$	5. Tension of uninhibited reflex when preceded by A = Y grm.	6. Amount of inhibition of reflex when preceded by A = Z grm.	7. $\frac{Z}{Y}$
I 32 σ A 9 σ E ..	244	133	0·55	228	131	0·57
I 41 σ A 0·9 σ E	247	119	0·48	208	173	0·83
I 41 σ E 5 σ A	271	133	0·49	90	82	0·91

(the relative amount of inhibition when E is preceded by A as well as by I) show, when compared with those of column 4 (the relative amount of inhibition when E is preceded by I only) that the proportion of the unresponsive motoneurones is greatly increased by an antidromic volley which reaches the motoneurones immediately before the reflex discharge is set up—at an interval of not more than 5 σ in observation 3. This is not so with observation 1, where the reflex discharge follows the antidromic volley by an interval sufficiently long for recovery from the refractory period. Thus in observations 2 and 3 the relative inexcitability due to the inhibition has been intensified by the relative inexcitability of the refractory period which follows the antidromic volley (Eccles, 1931). The inhibition has not prevented an impulse fired into a motoneurone antidromically from giving rise to a refractory period of the actual locus at which a reflex discharge is set up. Thus the passage of an antidromic impulse over the surface of a motoneurone is not appreciably affected by inhibition. *The failure of an antidromic impulse to affect inhibition cannot therefore be ascribed to a blocking of this impulse by the inhibited condition of the motoneurone.*

5. *The Effect of Excitatory Impulses on Inhibition.*

The comparatively long duration of the inhibited state of a motoneurone makes it possible to investigate any influence on the inhibition which may be exerted by excitatory impulses concurrently incident on that motoneurone. The experimental procedure is to examine the effect of an excitatory volley, E_1, interpolated between an inhibitory volley, I, and a second excitatory volley, E_2.

The following experimental conditions must be fulfilled before reliable results can be obtained :—

(1) The contralateral (inhibitory) volley must not have any appreciable excitatory admixture.

(2) The first ipselateral (excitatory) volley must not inhibit the reflex response to the second excitatory volley (*cf.* Eccles and Sherrington, 1931, *b*).

(3) The direct facilitation between the two excitatory volleys must not be of excessive duration. Such direct facilitation is a factor complicating any change which the first excitatory volley might make in the inhibition of the response to the second excitatory volley.

Throughout all series of experiments the conditions enumerated above have been tested for by numerous control observations.

Plate 9, fig. 5, shows part of a series of observations from one experiment. If observations 2 and 6 be compared with observations 1, 3 and 7, where E_1 was interpolated between I and E_2, the action-currents of the responses to E_2 show that E_1 diminishes the inhibitory effect which I exerts on E_2. Analysis of the mechanical responses confirms this finding, and supplies quantitative data for the extent of the diminution of the inhibition. Observations 4 and 5 show that, when the inhibition is absent, the size of the response to E_2 is unaffected by the precedence of E_1, *i.e.*, there is no inhibition or facilitation. In observation 5 the larger deflection of the action-current of the response to E_2 is due to the less asynchronous nature of the reflex discharge (Forbes and Gregg, 1915; Eccles and Sherrington, 1931, *a*), for analysis of the mechanical response shows that its magnitude is unaltered.

Many similar series of observations have been obtained in each of the seven experiments in which this observation has been carried out. Plate 9, fig. 6, shows part of a series from another experiment.

However, in one experiment with the preparation deafferented as usual, when several series of observations similar to those shown in Plate 9, figs. 5 and 6, had been obtained, a change occurred and subsequent series of observations showed that E_1 had very little, if any, effect in diminishing the inhibition of E_2. The reason for this change was untraced.

The adequacy of the observation employed to control the absence of a direct facilitation between E_1 and E_2 might be questioned, so a further control has been used. The difficulty is due to the possibility that the small inhibited response to E_2 may be directly facilitated by E_1 even when the uninhibited response shows no sign of facilitation. In Plate 9, fig. 7, observations 4 and 5, the stimulus setting up E_2 has been reduced so that the uninhibited response to

E_2 (observation 5) is of the same order of magnitude as the previous inhibited response to E_2 (observations 1 and 3). Observation 4 shows that then there is also no appreciable facilitation by E_1, and contrasts with observation 2 where, owing to the interpolation of E_1, the inhibited response to E_2 is greatly increased, *i.e.*, the inhibition of it is diminished.

It is therefore possible to conclude that the interpolated excitatory volley diminishes the inhibition of the response to a second excitatory volley even when it has no direct facilitatory effect on this response. There are two ways in which this could happen :—

(1) Some of the impulses of the interpolated excitatory volley may inhibit internuncial neurones in the inhibitory pathway (see fig. 1).

(2) Excitatory impulses may inactivate some of the inhibition when they are incident on an inhibited motoneurone.

Of these alternative possibilities the second has been accepted because it is simpler and does not involve the assumption of a set of impulses for which no other evidence exists. Experimental evidence (*v.i.* this section) also favours the second alternative.

In order to investigate further the nature of the effect of excitatory impulses on inhibition, E_1 has been interpolated at various times relative to I and E_2. A quantitative value for the effect of E_1 on the inhibition of E_2 by I has been calculated as in Table V.

A series of observations not shown in Table V gave a value of 128·5 grm. (average of four determinations) for the normal uninhibited response to E_2, and a value of 55 grm. (average of five values) for the response to E_2 when inhibited by I at an IE_2 interval of 118 σ. At this interval, which was kept constant throughout the series, the normal inhibition was therefore 73·5 grm. In column 3 are the tensions of the responses to E_2 when preceded by E_1 (calculated by the method of maximum tension differences (Eccles and Hoff, 1931)), and in column 4 are the ratios of these values to the tension of the response to E_2 alone (128·5 grm.). The ratios at the longer E_1 E_2 intervals are probably entirely dependent on the wave-summation of muscular contractions, but the higher values at the two shorter intervals indicate a facilitation of the response to E_2 by E_1 (Eccles and Sherrington, 1931, *b*). In column 5 are the tensions of the responses to E_2 when preceded by both I and E_1 (also calculated by the method of maximum tension differences). On the assumption that muscular summation and direct facilitation increase these values in the same ratio as they do the uninhibited responses to E_2 at similar E_1 E_2 intervals,

Table V.

1.	2.	3.	4.	5.	6.	7.	8.
Order of observation.	Stimulus intervals.	Maximum tension added to response to E_1 by E_2 in absence of inhibition = V grm.	Ratio of values of column 3 to maximum response to E_2 alone (128·5 grm.) = V/128·5 = W.	Maximum tension added to response to E_1 by E_2 in presence of inhibition = X grm.	Actual tension of inhibited response to E_2 calculated as X/W = Y grm.	Actual inhibition of response to E_2 = 128·5 − Y = Z grm.	Relative alteration by E_1 in the inhibition of response to E_2 = Z/73·5.
4	I 98 σ E_1 20 σ E_2	215 232		205 213 208			
		223·5	1·74	209	120	8·5	0·12
1	I 89 σ E_1 29 σ E_2	244 210		204 182			
		227	1·77	193	109	19·5	0·26
2	I 72 σ E_1 46 σ E_2	167 161		169 112 110			
		164	1·28	130	102	26·5	0·36
3	I 44 σ E_1 74 σ E_2	162 130		105 93·5 97			
		146	1·14	98·5	86	42·5	0·58

i.e., by the ratios shown in column 4, the tensions of the actual inhibited responses to E_2 are calculated by dividing the values of column 5 by those of column 4, and are shown in column 6. The actual amounts of the inhibitions are then calculated by subtracting these tensions from 128·5 grm., and they are shown in column 7, while in column 8 these inhibitions are expressed as a ratio of the normal inhibition (73·5 grm.).

When E_1 closely precedes E_2, *e.g.*, in the results of observation 4, the inhibition is reduced to only a small fraction of its former value, in this case to 0·12. The longer the E_1 E_2 interval, the smaller is the effect of E_1 on the inhibition of the response to E_2. Many similar series of observations have been obtained both in this and other experiments. Thus at a short interval after E_1 there is an almost complete disappearance of the inhibited condition of the motoneurones, and then there follows a gradual rebuilding up of the inhibition. This reappearance must be due to the incidence of fresh inhibitory impulses on the motoneurones. The presence of such delayed inhibitory impulses is also indicated by the intensity of the inhibition normally increasing for even as long as 80 σ after the setting up of an inhibitory volley (see fig. 2).

It was mentioned above that there was a possible alternative explanation of the diminution of the inhibition by the interpolated excitatory volley. Some of the impulses of the excitatory volley may inhibit internuncial neurones in the inhibitory pathway, and so stop the firing of inhibitory impulses on the motoneurones in question. Since the internuncial neurones are probably on the contralateral side, the temporal course of their inhibition, if present, is likely to follow a course similar to that found for inhibition of contralateral flexor motoneurones. There would then be a considerable period during which the diminution of the inhibition of the flexor centre by the interpolated excitatory volley would become more marked. Actually the diminution of the inhibition reaches its maximum much more rapidly, and in a few sigmata the inhibition is being built up again. The rapidity of the action of the interpolated excitatory volley argues for a direct effect by it on the inhibition of the motoneurones.

V. DISCUSSION.

An excitatory volley interpolated between an inhibitory volley and a second excitatory volley diminishes the inhibition of the latter when preceding it by even as long an interval as 100 σ. Some of the inhibition which was present on the motoneurones during the incidence of impulses from the first excitatory volley must, in the absence of that volley, persist long enough to inhibit the response to the second excitatory volley. But the impulses from the first excitatory volley are incident on the motoneurones for a relatively short period, for control observations show that the facilitation following the first excitatory volley is short in duration, *e.g.*, 20 σ to 30 σ. *Hence the inhibition of a motoneurone must be a state able to persist for a considerable period*, e.g., *at least* 50 σ *in the experiment quoted in Table V. The persisting inhibitory condition may be called a central inhibitory state, c.i.s., on analogy with the somewhat similar central excitatory state.*

The long duration of inhibition following a single inhibitory volley will in part be due to the persistence of the c.i.s. set up by each individual inhibitory impulse, but this will not account for the relatively gradual increase in the intensity of the inhibition which occurs for some time after its first onset (figs. 2 and 3). *This period of building up of the inhibition must be due to the summation of the separate inhibitions produced by each of the inhibitory impulses incident throughout the whole period.* The subsequent decline in the intensity of the inhibition shows that there is a gradual subsidence of the accumulated c.i.s.

Thus three factors at least are responsible for the temporal course of the inhibition following a single inhibitory volley :—

(1) Temporal dispersion of the inhibitory impulses incident on the motoneurones.

(2) Persistence of the central inhibitory state produced by each inhibitory impulse.

(3) Subsidence of the central inhibitory state.

It is now possible to describe the condition of the flexor motoneurones at various times after the setting up of an inhibitory volley.

An inhibitory volley is without effect on the reflex twitch evoked by an excitatory volley until the latter volley is set up about 8 σ later (see § 2). Thus, on the assumption that a relatively insignificant time is taken by an inhibitory impulse to give rise to c.i.s., there is an interval of about 16 σ between the setting up of the inhibitory volley and the incidence of the first inhibitory impulse on the motoneurones. If the rate of travel of inhibitory impulses is similar to that of excitatory impulses, *i.e.*, about 35 metres a second (Eccles and Sherrington, 1931, *a*), about 4 σ will be accounted for by the time of travel in the peripheral afferent nerve. This leaves 12 σ for the time of travel within the spinal cord of the fastest inhibitory impulses.

The increase in the intensity of the inhibition for 20 σ to 50 σ after its onset (figs. 2 and 3) shows that there is a continuous heavy bombardment of the motoneurones by inhibitory impulses with the resulting summation of the c.i.s. produced. Presumably during this period there is some subsidence of the c.i.s. already produced, but it is not apparent till the inhibitory bombardment slackens. Then momentarily the rate of production of c.i.s. by the incidence of fresh impulses equals the rate of subsidence of the preformed c.i.s. This is the instant of maximum inhibition. Thereafter the subsidence is more and more in the ascendency. But the bombardment by inhibitory impulses continues for some time after the instant of maximum inhibition, and it becomes apparent if the previous inhibition is removed by an excitatory volley, *e.g.*, in the observations of Table V, c.i.s. was built up after an excitatory volley which was set up almost 100 σ after the inhibitory volley.

However, a sufficiently late portion of the inhibitory curve is likely to be almost uncomplicated by the production of fresh c.i.s. In some experiments this part of the curve has approximated to the exponential form found by

Bremer (1931), *e.g.*, in fig. 4, after a tetanic inhibition. But the ordinates (isometric tension values for the inhibition) can at best be considered as proportional to the numbers of inhibited motoneurones. It has further to be assumed that there is an even distribution of the whole range of relative excitation-inhibition intensities in these motoneurones, before the exponential shape of the curve can be interpreted as showing that the rate of subsidence of c.i.s. is, at any instant, proportional to its intensity.

The above description of the events following an inhibitory volley may seem to entail an inhibitory bombardment of unexampled duration. But even with the spinal flexor reflex after-discharge following a single excitatory volley may last as long as half a second (Eccles and Sherrington, 1931, *c*), and during all this period the motoneurones are being bombarded by excitatory impulses at a rate sufficient to raise repeatedly the c.e.s. to threshold.

At first thought it is difficult to imagine the up-stream mechanism responsible for converting a single volley of impulses into these prolonged bombardments. However, such is the intricacy of the fibre system of the grey matter, as revealed by microscopic examination, that it would seem to be difficult to over-estimate its potentiality for providing "delay paths" (Forbes, 1922) adequate for the most prolonged of bombardments. The rate of conduction along the fine nerve fibres of the grey matter is probably even slower than the slowest rate found by Erlanger and Gasser (1930) in the non-medullated fibres of a cat's peripheral nerve—0·25 metres a second or 250 μ a sigma.

The time occupied by the various "synaptic" delays (Eccles and Sherrington, 1931, *a*) must be added to the total conduction time of the pathway. The shortest "synaptic" delay for the single synapse in the reflex arc of the ipselateral flexor reflex averages about 4 σ in duration, but "synaptic" delays of twice this duration are common. Thus, if there is only one synapse in the shortest inhibitory pathway, a considerable part of the time (about 12 σ, see above) occupied in this pathway is likely to be due to "synaptic" delay. This is also probably the case with more complicated inhibitory pathways, for in them there will be several synapses.

In view of the apparent facility with which c.i.s. can be summed, the additional excitation produced by the combination of two inhibitory volleys may seem disappointingly small. But it must be remembered that the inhibitory field does not extend over the whole flexor centre (Creed and Eccles, 1928). One inhibitory volley by itself inhibits all those motoneurones in the centre of the inhibitory field. A second inhibitory volley in addition inhibits those motoneurones in which an effective inhibition results from summation of the latent inhibitions produced by each volley alone. Similarly a repetitive series of inhibitory volleys inhibits a gradually increasing number of motoneurones, but limits are set by the restricted area of the inhibitory field.

The grading of the intensity of both the excitation and the inhibition of a single motoneurone has presented a difficulty to the hypothesis put forward by Lucas (Lucas, 1917; Adrian, 1924) in an attempt to explain inhibition as a type of Wedensky effect. Recent experiments by Forbes, Davis and Lambert (1930) have shown that, with the extensor motoneurone also, the stronger its excitation the more difficult it is to inhibit. By an ingenious line of discussion they point out that this result need not render the Lucas hypothesis untenable. However, if the inferences drawn from the present series of experiments are correct, namely, that the inhibition produced by any particular impulse persists for as long as 50 σ (see above), and that excitation and inhibition mutually inactivate each other, then an explanation in terms of the Wedensky effect appears to be impossible.

According to the Lucas hypothesis the inhibitory effect of each individual inhibitory impulse has a duration of the same order as the refractory period of nerve. The evidence for such a very short duration has been derived solely from the important experiments performed by Beritoff (1914, 1924), who states that the duration of a single inhibitory effect is not more than 4 σ.

In the experiment from which this value was derived the semitendinosus was reflexly excited by stimulation of the ipselateral peroneal nerve at a rate of 250 a second and inhibited by stimulation of the contralateral peroneal nerve at 125 a second. Although the electrical response of the muscle had a rhythm of 250 a second, its irregularity showed that the individual motoneurones were not discharging at this high rate (*cf.* Fulton, 1926, p. 347). During certain phases of the inhibition the rhythm of the response was reduced to 125 a second, and this was interpreted as showing that there was an inhibition of the second response of each motoneurone—hence the value of 4 σ for the duration of the inhibition. Since the individual motoneurones were not initially discharging at 250 a second, this value for the duration of the inhibition must be too small. Why, then, does the rhythm tend to be 125 a second during the inhibition? Before the inhibition most of the motoneurones probably responded to every second stimulus only, *i.e.*, at 125 a second, and the inhibition, which was almost total, apparently prevented all discharge from those motoneurones responding to one of the alternate series of stimuli. The result is that the few motoneurones still uninhibited show a rhythm of response of 125 a second. This selective effect of the inhibition is probable because alternate make and break shocks were used to elicit the reflex. The resulting differences in the sizes of the alternate excitatory volleys would account for the responses evoked by one alternate series partly surviving an inhibition which entirely abolished the other alternate series. No conclusion concerning the duration of inhibition can therefore be drawn from such experiments.

Experiments with a slower rate of excitation, *e.g.*, 100 a second (Beritoff, 1924), are not open to the above criticism, but the durations of the inhibitions under such conditions (20 σ or more) have been so long that it has been thought necessary to postulate the existence of a repetitive series of the short inhibitions (Beritoff, 1924). The duration of inhibition with an actively excited motoneurone will naturally be much shorter than with a resting motoneurone, for in the former the inhibition will be inactivated by the continuously arriving excitatory impulses. Under such circumstances the effective duration of the inhibition will be the period during which inhibitory impulses are still arriving.

The duration of the inhibitory periods in Beritoff's experiments with excitatory rhythms of 100 a second or less (see also Adrian, 1924) gives, therefore, no indication of the duration of any individual unit of the inhibition. The interesting experiments of Brucke (1922) fail to throw light on the duration of the inhibitory process for the same reason.

From the long duration of the inhibition set up by a single inhibitory volley, Samojloff and Kisseleff (1927) inferred that there is a long lasting inhibitory state in the reflex centre, and they suggested that it is a chemical substance of opposite nature to the "excitatory substance." In their discussion they do not take into consideration either the existence of internuncial neurones in the inhibitory pathway or the possibility of a significant temporal dispersion of the incident inhibitory impulses. They believed that the duration of the inhibited condition of the centre was due entirely to the persistence of an "inhibitory substance." A similar explanation has been suggested for the long-lasting inhibition of the spinal knee jerk by a single ipselateral volley (Ballif, Fulton and Liddell, 1925; Fulton, 1926). The simpler nature of the inhibitory pathway with such ipselateral inhibition makes it probable that the temporal dispersion of the inhibitory impulses has less relative significance than is the case with centralateral inhibition. However, in this connection it is well to remember that ipselateral flexor inhibition has a temporal course very similar to contralateral inhibition (Eccles and Sherrington, 1931, *b*). Ipselateral flexor inhibition has, however, a shorter latent period.

Two types of experimental treatment of central inhibitory state have been attempted with totally different results :—

(1) *An antidromic impulse has no effect on c.i.s., but it is followed by its normal refractory period as is shown by the transient accentuation of the relative inexcitability of the motoneurone.*

(2) *Excitatory impulses incident on a motoneurone inactivate c.i.s. At the same time these excitatory impulses do not give rise to effective c.e.s. for, if they did, a discharge would be set up after the c.i.s. had been inactivated* (*see* § 5). *Thus it seems that the c.e.s. and the c.i.s. suffer a mutual inactivation.*

The rate of production of c.e.s. by incident excitatory impulses controls the reflex discharge of reflex impulses from a motoneurone (Eccles and Sherrington, 1931, *c*). Inhibition modifies this response of the motoneurone to excitation, not by any direct action on the motoneurone itself (*cf.* Sherrington, 1929), but by inactivating c.e.s. with the result that the rate of increase of c.e.s. is slowed down or stopped altogether.

The locus of the motoneurone from which reflex discharge is set up (probably the synapse, see Eccles and Sherrington, 1931, *d*) is open to approach from three different types of impulse, the excitatory impulse, the inhibitory impulse, and the antidromic impulse. Each of these is followed by a characteristic condition: c.e.s., c.i.s., and refractory period, respectively. In addition it has already been shown that an antidromic impulse inactivates c.e.s. (Eccles, 1931), and in the present series of experiments an excitatory impulse, or the c.e.s. it gives rise to, has been shown to inactivate c.i.s. And finally c.i.s. and therefore the inhibitory impulse probably inactivates c.e.s. Yet in spite of this interaction which exists between two pairs of the three types of impulses, an antidromic impulse has no action whatever on c.i.s.

In many respects c.e.s. and c.i.s. are analogous states, but they differ fundamentally in certain features. In the following respects c.e.s. and c.i.s. are analogous :—

(1) c.e.s. and c.i.s. are only produced when nerve impulses in the terminal branches of one neurone are incident on a neurone next in series, *i.e.*, at synapses. However, there is yet no experimental evidence for the existence of inhibition with neurones other than motoneurones.

(2) The c.e.s. or c.i.s. produced by a single impulse undergoes a gradual subsidence. With flexor motoneurones the rate of subsidence for c.e.s. (Eccles and Sherrington, 1930, *a*) is several times more rapid than the rate for c.i.s.

(3) The c.e.s. or c.i.s. produced by one impulse sums respectively with the c.e.s. or c.i.s. produced by other impulses arriving by either the same or other nerve fibres ending on that same motoneurone. It may be that there is only summation between the c.e.s. or c.i.s. produced at immediately adjacent synapses.

(4) As a consequence of summation many grades of intensity of either c.e.s. or c.i.s. may be produced on a motoneurone.

(5) When c.e.s. and c.i.s. are opposed to each other, there is an inactivation of certain quantities of each. This mutual inactivation is likely to be quantitative, in which case there is a true algebraic summation between the c.e.s. and the c.i.s. of a motoneurone. It follows that c.e.s. and c.i.s. cannot coexist in the same place for any appreciable time. It may be that there is a quantitative interaction between the c.e.s. produced by one excitatory impulse and the c.i.s. produced by one inhibitory impulse, but such a relationship is at present pure hypothesis.

In the following respects c.e.s. and c.i.s. differ fundamentally :—

(1) If the c.e.s. of a motoneurone reaches a sufficient intensity, then it gives rise to the discharge of an impulse from that motoneurone. No corresponding action is known for c.i.s. It has no direct effect on the motoneurone—it merely inactivates c.e.s.

(2) An impulse passing antidromically up a motor nerve fibre to a motoneurone inactivates the c.e.s. of that motoneurone, but does not affect the c.i.s.

The above two differences between c.e.s. and c.i.s. have in all probability a common basis. *Thus it may well be that c.e.s. acts as the sole intermediary between c.i.s. and the motoneurone, c.i.s. having no direct action on this latter.*

None of the experimental evidence bears on the actual nature of c.i.s. Ballif, Fulton and Liddell (1925), Fulton (1926), and Samojloff and Kisseleff (1927) have suggested that it is a chemical substance. It may, however, receive its ultimate explanation in terms of physical chemistry, *e.g.*, as the stabilising of a surface membrane (Sherrington, 1929). Certain it is that it can exist independently of excitation.

VI. SUMMARY.

The inhibition of flexor motoneurones by a single contralateral volley has been investigated by observing the reflex response evoked by a single ipse-

lateral volley set up at various times after the contralateral volley (*cf.* Samojloff and Kisseleff). A reduction in the size of the reflex response, *i.e.*, inhibition, is not usually observed until the excitatory volley follows the inhibitory by 8 σ or more. After its first appearance the amount of the inhibition increases for 25 σ to 70 σ and then progressively decreases, but is still usually observable at 200 σ.

In some experiments a given inhibitory volley has inhibited a weaker reflex more than a stronger. Consequently it is concluded that with motoneurones the prevention of reflex discharge by inhibition depends on the intensity of the excitation.

Two similar inhibitory volleys in the same afferent nerve produce a greater inhibition than either volley alone, even when the interval between them is as long as 60 σ. Further, a repetitive series of inhibitory volleys produces a gradually increasing inhibition. Both these results show that there is summation of the inhibitory effects of successive afferent volleys.

A volley passing antidromically up motor nerve fibres to inhibited motoneurones has no effect on that inhibition.

Excitatory impulses incident on inhibited motoneurones diminish the inhibition of a reflex evoked by a subsequent excitatory volley. Hence it is concluded that inhibition is a long-lasting state, called a central inhibitory state, and that it is inactivated by excitatory impulses. In order to explain the results it must further be assumed that the inhibitory impulses from a single inhibitory volley are incident on motoneurones during a considerable period. This temporal dispersion, often at least 100 σ in duration, is attributed to delay paths.

In the discussion there is a detailed treatment of the central conditions set up in the above experimental procedures. It is suggested that the central inhibitory state has no direct effect on the motoneurone, its effect being entirely due to the mutual inactivation resulting when it and the central excitatory state interact.

It would seem that some of the inferences drawn from the experimental evidence render untenable explanations of inhibition based on the Wedensky effect.

One of us (J.C.E.) wishes to express his gratitude to the Medical Research Council for a personal grant, and we have to thank the Royal Society for a grant towards the apparatus used in this research.

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EXPLANATION OF PLATES.

PLATE 8.

FIG. 1.—Reflex responses of deafferented tibialis anticus muscle when an inhibitory volley, I, in the contralateral peroneal nerve, is set up at various intervals before an excitatory volley, E, in the ipselateral popliteal nerve : 1, I 41σ E ; 2, I 80σ E ; 3, I 200σ E ; 4, I 121σ E ; 5, E alone ; 6, I 163σ E ; 7, I 92σ E. In this and subsequent figures the white lines of the myograph record commence *below* the dark lines of the corresponding electrical record. Time, 1 d.v. = 10σ in all records. Tension scale at side.

FIG. 2.—Reflex responses of deafferented tibialis anticus muscle when either one or both of two inhibitory volleys, I_1 and I_2, in the contralateral peroneal nerve, are set up at constant intervals before an excitatory volley, E, in the ipselateral popliteal nerve :

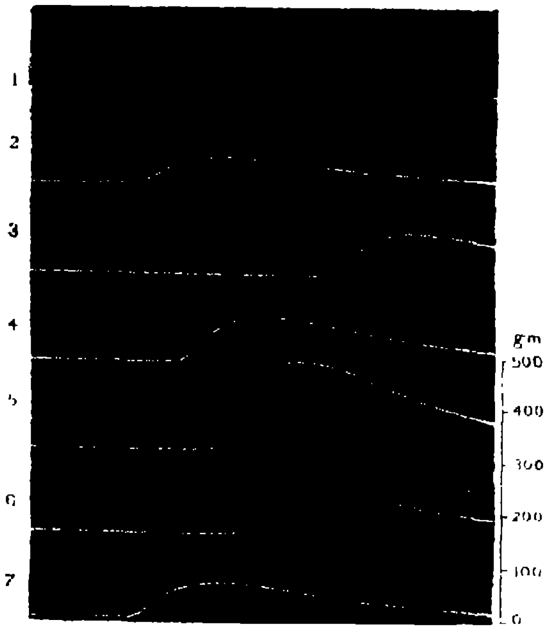

FIG. 1.

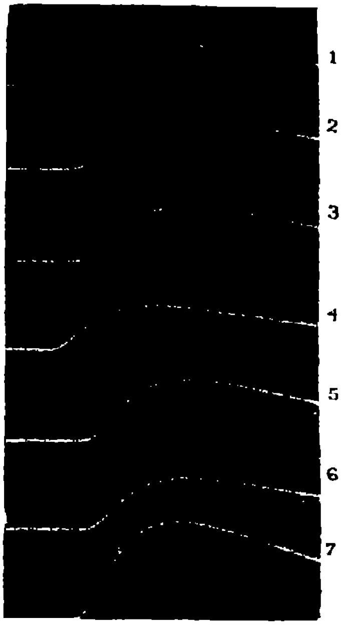

FIG. 2.

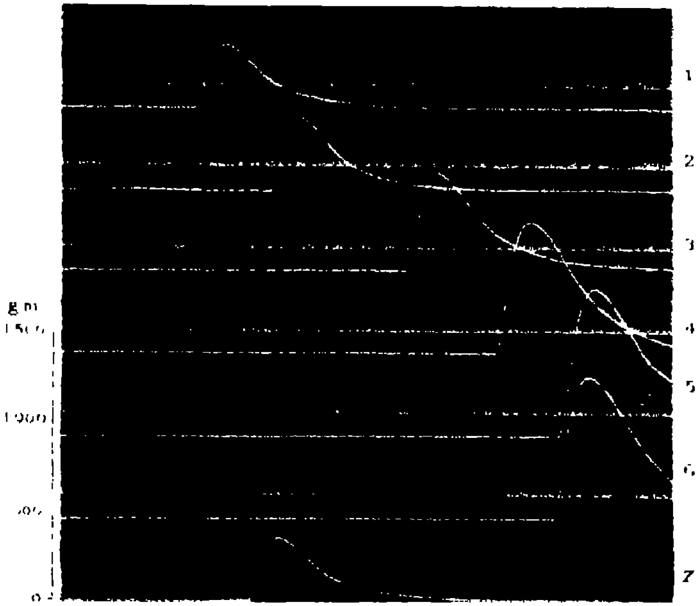

FIG. 3.

(*Facing p.* 112.)

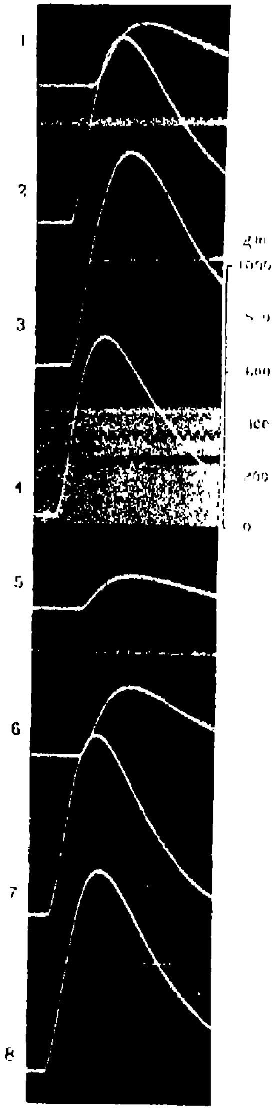

Fig. 4.

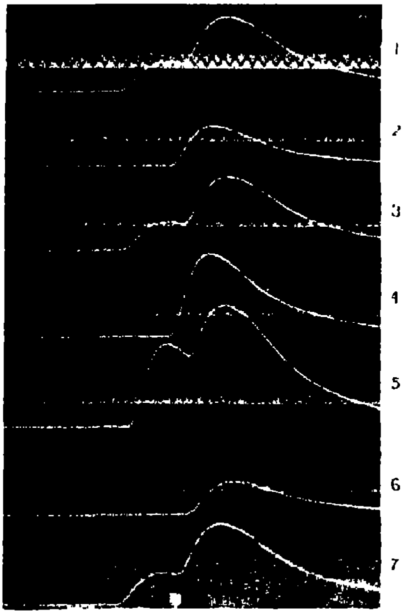

Fig. 5.

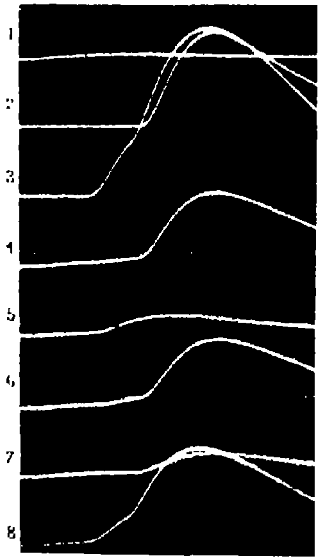

Fig. 6.

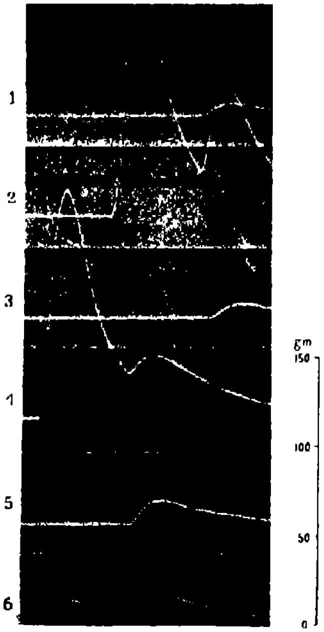

Fig. 7.

1, I_1 39σ, I_2 59σ E (no accompanying electrical response); 2, I_2 59σ E.; 3, I_1 98σ E; 4, I_1 39σ, I_2 59σ E; 5, I_2 59σ E; 6, I_1 98σ E; 7, E alone. Tension scale as for Plate 8, fig. 1.

FIG. 3.—Reflex responses of deafferented tibialis anticus muscle evoked by an excitatory volley, E (in the ipselateral popliteal nerve) at various times during and after tetanic stimulation of an inhibitory nerve, the contralateral peroneal. The intervals before or after the cessation of the tetanic stimulation (shown by the signal line above the corresponding electrical record) were as follows: 1, 11σ before end; 2, 78σ after end; 3, 239σ after end; 4, 345σ after end; 5, reflex alone; 6, 424σ after end; 7, 52σ after end. Tension scale at side.

Plate 9.

FIG. 4.—Responses of deafferented tibialis anticus muscle elicited by a single maximal stimulation of its intact motor nerve, *i.e.*, an antidromic volley, A, interpolated between an inhibitory volley, I, in the contralateral popliteal nerve, and an excitatory, E, in the ipselateral posterior tibial nerve as follows: 1, E alone; 2, I 104σ A, 14σ E; 3, A 14σ E; 4, A alone (the small late action-current is an artefact); 5, I 118σ E; 6, E alone; 7, I 113σ A, 5σ E; 8, A 5σ E. Tension scale at side.

FIG. 5.—Reflex responses of deafferented tibialis anticus muscle when an inhibitory volley, I, in the contralateral peroneal nerve precedes excitatory volleys, E_1 and E_2, in the ipselateral popliteal nerve as follows: 1, I 59σ E_1 59σ E_2; 2, I 118σ E_2; 3, I 59σ E_1 59σ E_2; 4, E_2 alone; 5, E_1 59σ E_2; 6, I 118σ E_2; 7, I 59σ E_1 59σ E_2. Tension scale as for Plate 9, fig. 4.

FIG. 6.—Reflex responses of a not quite completely deafferented tibialis anticus muscle when an inhibitory volley, I, in the contralateral peroneal nerve, precedes excitatory volleys, E_1 and E_2, in the ipselateral hamstring nerve as follows: 1, I alone; 2, E_2 alone; 3, E_1 28σ E_2; 4, I 41σ E_1 28σ E_2; 5, I 41σ E_1; 6, I 41σ E_1 28σ E_2; 7, I 69σ E_2; 8, I 41σ E_1 28σ E_2. Tension scale as for Plate 8, fig. 1. A small reflex response is evoked by the contralateral volley.

FIG. 7.—Reflex responses of a deafferented tibialis anticus muscle when an inhibitory volley, I, in the contralateral peroneal nerve, precedes excitatory volleys, E_1 and E_2, in the ipselateral popliteal nerve. After observation 3, E_2 was made weaker, and for observation 6, E_1 was also weakened. 1, I 118σ E_2; 2, I 0·0σ E_1 118σ E_2; 3, I 118σ E_2; 4, E_1 118σ E_2; 5, E_2 alone; 6, E_1 118σ E_2. Tension scale above. Electrical record shows interference by A.C.

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A Simple Method of Measuring Surface Tension.

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It is known that the surface tension of solutions of many hydrophilic colloids such as proteins, saponins and soaps, is comparatively high when the surface is newly formed and that it falls rapidly at first and then more slowly (du Noüy, 1919, 1926; Wilson and Ries, 1923; Harkins and Zollmann, 1926). This fall may continue for many hours and measurements of the surface tension of such a solution cannot therefore have much significance unless either the surface has reached equilibrium or the age of the surface is known.

The method which is most frequently used for the study of the surface tension of such solutions is that which depends on the measurement of the force exerted by the liquid on a wire ring which is placed on the surface and then pulled away (du Noüy, 1926; Harkins and Jordan, 1930). It is the purpose of this paper to describe a simpler method, which is accurate enough for many purposes and which is especially suitable for these studies. Certain curious reactions of the surface of solutions of saponin to stretching will also be described.

In discussing these observations the term "surface tension" is taken to mean the total apparent tension due to the surface phase, though there is evidence that in some cases this phase may extend through many molecular layers.

Method.

The method is a modification of the well-known method which depends on the measurement of the weight of drops. This has been found to give very accurate and consistent readings with pure liquids, and owing to its simplicity it has been widely used for measurements on various complex solutions.

Since the size of the drops of complex solutions may be greatly affected by the rate at which they are formed, those who use this method frequently abandon all attempt at absolute measurement and are content to compare the number of drops produced by a known volume of one liquid with that produced by the same volume of another, when both drop in the same time from the same nozzle.

It has been found that the micrometer syringe (Trevan, 1925) provides a convenient and accurate method of measuring the size of a single drop. In this instrument the position of the piston of a glass syringe is controlled by a micrometer screw, the rotation of which through one division causes the syringe to deliver 0·2 c.mm. In this way the volume of the drop is under very good control, and continuous stretching of the surface can be avoided. A nozzle with a circular tip of suitable diameter is fitted to the syringe, which is then filled with the liquid whose surface tension is to be studied. In the study of solutions of substances which produce a slow fall of surface tension the micrometer head is rotated rapidly through a known number of scale divisions so that a known volume of liquid is made to hang from the tip. If the drop is large enough, it will fall after an interval of time which depends on its weight and volume. If the diameter of the tip is known the value of the surface tension at this moment can be calculated from the volume of the drop as measured by the micrometer syringe, and the density of the solution.

Nozzles made of glass and ground so as to fit on to the end of the micrometer syringe have been used, but it is more convenient to use a nozzle turned out of brass and soldered on to the fitting removed from one of the needles provided for use with the syringe. The diameter of the hole through which the liquid flows should be small compared with the external diameter of the tip. Harkins and Brown (1919) have shown that the volume of a drop falling from a brass ring is the same as that of one falling from a glass ring of the same diameter. Accurate brass nozzles can, of course, be more easily made than glass ones and are less liable to be damaged by use, but they cannot be cleaned with strong acids. However, prolonged washing with running tap-water is sufficient to remove the impurities which interfere with the determination of the surface tension of watery solutions by this method. It has been suggested that nozzles might be made of platinum in order to facilitate cleaning. This precaution has not been found necessary in the present work.

The most satisfactory results have been obtained with a nozzle having a tip with an external diameter of approximately 2 mm. If nozzles much larger than this are used the drop is not formed so regularly and the results obtained are more dependent on the exact rate at which the drop is formed. The syringe must, of course, be fixed rigidly in a strictly vertical position and be free of vibrations. The drop must be formed at a known temperature in a small closed vessel, so that it is protected from draughts and from evaporation.

There is no simple mathematical relation between the size of the drop which falls and the surface tension (Rayleigh, 1899; Freud and Harkins, 1929). The surface tension may, however, be calculated from the formula

$$\gamma = mgF/r,$$

where γ is the surface tension, m is the mass of the drop which falls, g is the acceleration due to gravity, r is the radius of the nozzle, and F is a constant depending on the shape of the drop (Young and Harkins, 1928). The appropriate value of F is obtained by calculating V/r^3 (where V = the volume of the drop which falls), and referring to a table. The nozzles found suitable for the experiments described in this paper were smaller than those generally used, but the appropriate values of F are known with sufficient accuracy for most purposes. The values of the surface tension given in Table I have been calculated in this way in order to simplify the procedure. In these calculations the value of log F was obtained by linear interpolation in a table connecting log F and log (V/r^3). It is thought that, for the large values of F with which we are concerned, this method of calculation is especially accurate.

Table I.—Showing the surface tension in dynes per centimetre for drops of different volumes, and for different values of $\log_{10} r$, where r cm. = radius of nozzle.

$\log_{10} r$.	Volume of drop in cubic centimetres.				
	0·01.	0·02.	0·03.	0·04.	0·05
$\bar{1}\cdot 0$	23·6	44·9	65·6	86·2	106·5
$\bar{1}\cdot 05$	21·6	41·0	59·8	78·3	96·7
$\bar{1}\cdot 1$	19·7	37·5	54·5	71·3	87·9
$\bar{1}\cdot 15$	17·9	34·3	49·9	65·1	80·0

This table gives the surface tension in dynes per centimetre for a liquid of unit density, where $g = 981\cdot 3$. For other liquids these values must be multiplied by the density of the liquid. By giving values of $\log_{10} r$ instead of values of r, the maximum error which occurs in calculating intermediate values by simple linear interpolation has been reduced to about 0·1 dyne per centimetre. It is easy to prepare from this table a curve for any particular nozzle connecting the volume of the drop with the surface tension. These curves are nearly, but not quite, linear.

Iredale (1923) gives another method of calculation based on a slightly different theory, which gives the same results as the above method.

RESULTS.

Surface Tension of Pure Liquids.

As a check on this method, measurements were made on various standard liquids whose surface tensions are known, and independent of the age of the surface. For this purpose the piston of the syringe was screwed down rapidly until the drop was almost large enough, and the last cubic millimetre was added very slowly and carefully until the drop fell. When this occurred the movement was stopped, and a reading was taken. The difference between two successive such readings was taken as the volume of the drop that had fallen, the volume of liquid left hanging on the nozzle being assumed to be constant.

Ten successive such readings each of the volume of a single drop gave values for the surface tension of distilled water at 26° ranging between 71·7 and 72·3 dynes per centimetre. The value given by Young and Harkins (1928) is 71·82.

A comparison of the results obtained by this method with the accepted standard values (Young and Harkins, 1928) is given in Table II.

Table II.

Substance.	Temperature.	Surface tension dynes per cm.	Standard values.
	°		
Water	26	72·0	71·82
4 molar NaCl in water	23	79·2	78·8
Toluol	15	29·1	29·0
Nitrobenzene	23	42·7	43·9

The agreement between the results was considered sufficiently close. The discrepancy with nitrobenzene was possibly due to the presence of impurities, since no special precautions were taken to ensure the purity of the preparation.

Surface Tension of Solutions of Surface Active Substances.

In the case of these pure liquids the surface tension is, within wide limits, independent of the age of the surface, and it was found that a drop only slightly smaller than that which fell would remain hanging on the apparatus for an indefinite time, but in the case of watery solutions of various surface-active substances this was not the case. A small drop of a solution of saponin may be almost spherical when it is first formed, but as the saponin passes to the surface and the surface tension diminishes, the drop slowly lengthens out and eventually, if it is large enough, it will fall off. By measuring the time required by rapidly formed drops of various sizes to fall it is a simple matter to obtain the relation between the surface tension and the age of the surface.

Measurements have been made of the volume of drops by withdrawing the residual liquid after the fall of each drop, but more consistent values have been obtained by taking the reading immediately and by assuming, as before, that the volume left hanging on the nozzle is constant. A definite error is, of course, introduced by this assumption, but this error is too small to be detected in experiments carried out in this way.

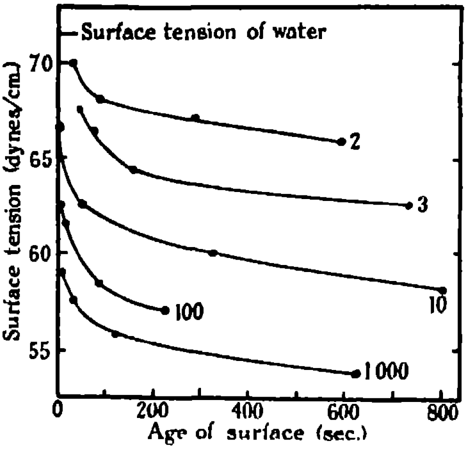

Fig. 1.—Measurements of surface tension of saponin (Merck) solutions at different times after the formation of the surface. Figures attached to curves give concentration of saponin in parts per 50,000. Temperature 18·5° C.

Some measurements of the surface tension of different dilutions of a solution of saponin (Merck) are shown in fig. 1. It will be seen that the surface tension fell rapidly in the first few seconds, and had not reached an equilibrium after 10 minutes. Similar results have been obtained with a preparation of bile salts, with rabbit serum, cat serum, and with a broth culture-medium. The curves are very similar to those published by du Noüy (1926). Since the range of times covered is rather wide it is convenient to plot the surface tension against the logarithm of the time. In the case of saponin solutions it has invariably been found that the results, plotted in this way, showed only small and irregular deviations from a straight line. In other words, the fall of surface tension was expressed with satisfactory accuracy by the equation $\gamma = A - B \log t$ (where γ is the surface tension and t the time), and A and B are constants. The fall of surface tension of the other solutions mentioned above was equally well expressed by the same equation, which expresses the facts at least as accurately as that proposed by du Noüy (1926) and is simpler. The fall of tension in soap solutions appears to follow a rather different course, and to reach equilibrium comparatively rapidly. By plotting the surface tension against the logarithm of the time it is easy to deduce the surface tension at any given time by interpolation; but since it has not been found possible to discover any simple explanation of the equation it is doubtful whether it has any fundamental significance. It is probable that the fall of surface tension is governed by the laws of diffusion, and that its course could be expressed accurately only by a much more complex equation.

Effects of Changes of the Area of the Surface of Saponin Solutions.

The method described above gives a quantitative measure of the surface tension of the solution at the moment when the drop falls. It has been found that the surface tension of solutions of saponin shows reactions to changes in the area of the surface which cannot be followed in this way, but which are shown by the changes which occur in the shape of the drop. When the surface tension is high the drop will be nearly spherical, but when it is low the drop will be longer and narrower. The slow lengthening of a newly formed drop as its surface tension falls has already been mentioned. Some other reactions of the drop will now be described. A 1/500 solution of saponin (Merck) in water was used in all these cases, but the different experiments are not all strictly comparable with one another, since they were carried out at room temperature, and this was different on different days. In some cases the shape of the drop was observed directly; in others an enlarged image of the drop was thrown on a screen. The radius of the nozzle on which the drops were formed was 0·0967 cm.

Increasing the Area.—If the volume of a drop of saponin solution which has been formed for some minutes is suddenly increased by means of the syringe, the surface is stretched and the concentration of saponin therein must be diminished. It might be predicted that in these circumstances a rise in surface tension would occur, and this indeed appears to be the case. The drop immediately becomes more nearly spherical, and its total length may decrease although its volume is increased. After this rapid increase the surface tension again decreases, and the drop lengthens out and may eventually fall. The total age on falling, of the large drop thus formed in two stages, is greater than that of a drop of the same size formed rapidly in one stage. In the same way, if sufficient solution is added to a drop to cause it to fall immediately, the surface tension calculated from the size of this drop is larger than the static surface tension of a surface of the same total age measured by the method given above.

In measuring surface tension drops are sometimes allowed to hang for some time to attain equilibrium and then finally their volume is increased till they fall (Harkins and Brown, 1919). This procedure may be safely used for pure liquids, but the above observations show that it gives a value for the surface tension of saponin solutions that is larger than the true static surface tension.

When a surface is being stretched at a steady rate the stretching tends to raise the surface tension and, at the same time, the diffusion of saponin solution tends to lower it. If the stretch is a rapid one the total effect is, as we have seen, to raise the surface tension. If the stretch is infinitely slow the surface tension will continue the fall which was occurring before the stretching started. It is to be expected that with intermediate rates of stretching, the behaviour of the surface tension will be intermediate between these two extremes and that the surface tension in a rapidly stretching surface will, other things being equal, be higher than that in a slowly stretching surface. This conclusion is probably confirmed by the fact that when the saponin solution is caused to flow at a steady rate until the drop falls the size of the drop is increased by increasing the rate of flow. Thus, in one experiment, the volume of a drop formed in 66 seconds was 0·0280 c.c., while that of one formed in 11 seconds was 0·0294 c.c., and drops formed at intermediate rates had intermediate volumes. This phenomenon is known to occur with many surface-active solutions (Berczeller, 1914; Traube and Somogyi, 1914; Wolf, 1923).

When stretching ceases, the rate of fall of surface tension depends on the rate at which the stretch has occurred. After a rapid stretch the fall is apparently more rapid than after a slow stretch. In one experiment drops of 0·026 c.c. formed in about 5 seconds fell 45 and 47 seconds later, while a drop of the same volume formed slowly (in 24 seconds) did not fall till 76 seconds after its formation was complete. During the stretch the surface tension was presumably higher in the quickly formed drop, but when stretching ceased the surface tension fell so rapidly in this case that it reached the arbitrary level represented by the volume of the drop sooner than in the case of the slowly formed drop.

This somewhat paradoxical phenomenon has been repeatedly seen under conditions similar to those described above. It is well shown in the following experiment. When the micrometer head was rotated at such a rate that the volume of the drop increased at the rate of 0·0002 c.c. per second, the drop fell after 2 minutes 25 seconds, so that its volume was 0·029 c.c. A drop of 0·028 c.c., however, formed in about 5 seconds, fell within a second of the time when its formation was completed. Since this second drop was smaller than the first the surface tension was lower after 6 seconds than that of the other drop had been after 145 seconds.

Decreasing the Area.—If some of the liquid be rapidly withdrawn from a drop of saponin solution by means of the syringe the surface area is diminished and the concentration of saponin in the surface must be, at any rate momentarily, increased. Under these circumstances the surface tension falls; the girth of the drop diminishes considerably, but its total length may be actually increased. If sufficient fluid is withdrawn the surface tension reaches so low a value that a portion of the drop falls off. If the movement is made very rapidly, very small drops may be made to fall, and it seems probable that the surface tension of a rapidly contracting surface may fall to zero, or lower. If none of the liquid falls, the long thin drop slowly contracts up and becomes more nearly spherical. These changes in the shape of the drop are shown in fig. 2. They may be explained on the assumption that the diminution of the surface has increased the concentration of saponin beyond its equilibrium value and that

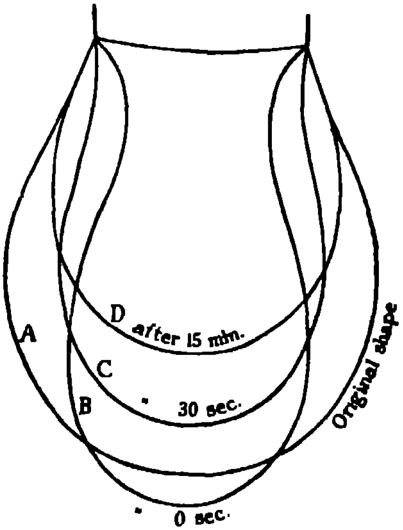

Fig. 2.—1/500 saponin solution. Diameter of tip, 0·0967 cm. Shape of image of drop thrown on a screen: A, drop, of volume 0·0182 c.c., after hanging 2 minutes. B, same drop, immediately after its volume was reduced to 0·0094 c.c. C, same drop, 30 seconds later. D, same drop, 15 minutes later.

it diffuses slowly back into the solution as the surface tension rises again. On the other hand, if the sudden decrease in volume is only slightly less than that necessary to cause the drop to fall immediately, the drop may slowly lengthen out and fall after several seconds.

Discussion.

It will be seen that, in many ways, the surface of a saponin solution behaves like a viscous-elastic system of the kind discussed by Gasser and Hill (1926) and Levin and Wyman (1927). Thus, a fairly rapid increase or decrease in its length and breadth produces an immediate increase or decrease in its tension; these immediate changes in tension are followed by slow changes in the reverse direction; and a rapidly stretching surface has a larger tension than a slowly stretching surface. The effects of very rapid alterations of length have not been studied. These facts are in accord with the conclusions of Shorter (1906), who found that the surface of a saponin solution acted on a rotating disc like a viscous-elastic system.

A simple explanation of the rapid changes has been given above in terms of the changes which must necessarily occur in the concentration of saponin in the surface. It has been suggested that the slow changes are due to the diffusion of saponin. It is not necessary to postulate the formation of any structure capable of exerting forces, to explain these particular facts, but it is known that a definite structure does exist. Plastic skins form in the surface-phase of solutions of saponin and many other substances (Ramsden, 1904; Wilson and Ries, 1923) and are responsible for the formation of persistent froths. It is possible that the phenomena described above are due, in part at least, to forces acting in these skins.

The behaviour of the tension of the surface of these saponin solutions may also be compared with that of plastic bodies, such as a piece of stretched rubber or muscle. The analogy is made closer in this case by the fact that the equation which has been found convenient to express quantitatively the fall of surface tension with time is identical with that which gives most accurate expression to the fall of tension in a piece of stretched rubber (Whitby, 1920). A similar equation has been found to apply to the after extension of muscles (Langelaan, 1915). Since muscles contain large quantities of surface-active substances, it is possible that similar forces are acting in the after-extension of muscle to those controlling the tension of the stretched surfaces of saponin solutions. In neither case are the forces necessarily acting actually in the surface layer of the molecules. In both cases the slow changes of tension may be due to the diffusion of molecules which affect the tension.

Other Solutions.

It has been mentioned that the fall of surface tension in the newly-stretched surface of solutions of bile salts, serum and a broth culture-medium was found to follow a course similar to that of saponin solutions. With the crude methods used, these other solutions did not show most of the other reactions described, but nevertheless it is probable that the difference between saponin solutions and solutions of other surface-active substances is quantitative rather than qualitative. Many different hydrophilic colloids form plastic skins on their surfaces (Ramsden, 1904). The large size of rapidly forming drops compared with slowly forming ones is also characteristic of many surface-active solutions. In some experiments with cat serum, diluted to 10 times its volume with distilled water, it was found that the total age on falling of a drop which was formed at a slow steady rate was about 10 times that of a drop formed rapidly. In the same way the total age on falling of a drop formed in two stages was greater than that of a drop of the same size formed in one stage.

All these surface-active solutions thus have much in common, and it is possible that more delicate methods of observation will show that the surfaces of most surface-active solutions have the plastic properties that are so clearly shown by saponin solutions.

Some Sources of Error in the Measurement of Surface Tension.

Surface-active substances tend to be adsorbed on the walls of the syringe and during an experiment their concentration in the solution falls. This fact introduces an error in the results, since as time goes on drops of a given size tend to remain longer on the nozzle before they fall. This phenomenon must occur whenever a surface-active solution is kept in a vessel without stirring, and must thus cause an error in most methods of measuring the surface tension. This error is in these experiments increased by the fact that the content of the syringe is small, and thus has a large surface in proportion to its volume. The observed variation in the results due to this cause was usually not larger than 0·05 dynes per centimetre. Constant readings could, however, with some solutions only be obtained by filling the syringe 15 minutes or so before the readings were taken.

If the solvent evaporates from the drop the weight of drop which falls will be overestimated. If the drop absorbs moisture its weight will be under-estimated. It was found by actual weighing of the drop that no appreciable evaporation or condensation of water occurred in 15 minutes at room temperature if the nozzle was enclosed by a small glass vessel wetted with the solution under test. It would be tedious to attempt to measure by this method the surface tension of surfaces older than this, and the method is thus not well suited to the measurement of the equilibrium value. The measurement of this is, however, usually not necessary, and sometimes impossible, since equilibrium is not attained even after 4 days (du Noüy, 1926). When the surface tension falls to a minimum and then increases (Wilson and Ries, 1923) the later changes cannot, of course, be followed by the method described in this paper.

The slow lengthening of a rapidly formed drop must be associated with some change in its surface area, but this change cannot be large and it probably has little effect on the surface tension. The method can thus be considered for practical purposes a " static " method of measuring the surface tension of a surface of any age. The actual detachment of the drop is a comparatively rapid process which must entail sudden changes of surface tension. The surface tension on which the result depends, however, is probably the value in the practically static drop when it becomes unstable.

The formation of the drop has been carried out as rapidly as possible by hand (*i.e.*, in about 5 seconds). This crude method appears to give sufficiently uniform conditions for most purposes, though large alterations of the rate at which it is carried out have a definite effect on the subsequent rate of fall of surface tension.

When drops are allowed to form at a steady rate the tension in their surfaces, which are being constantly stretched, is higher than that in a surface of the same age which has been rapidly formed, and then left stationary. Its exact level will depend on the rate of stretching. In Traube's method (1910) an attempt is made to keep this constant by causing the liquid to flow at a constant rate. The rate of stretching of the surface, however, will depend on the shape and size of the drops and be different for different solutions. It is thus impossible to obtain absolute results in this way.

It is clearly preferable to use a method such as that described above, in which prolonged stretching of the surface is almost entirely avoided. The method is simple and gives an absolute figure for the tension in a surface of any age.

Summary.

(1) The surface tension of a liquid can be determined in absolute units by measuring the volume of drops by means of the micrometer syringe.

(2) The slow fall of "static" surface tension in the newly-formed surface of a solution of a hydrophilic colloid can be followed by this method.

(3) The apparent viscous-elasticity of the surface of saponin solutions is clearly shown by the changes which occur in the shape of drops. The rapid changes can be explained in terms of the changes which must occur in the concentration of saponin in the surface. It is suggested that the slow changes are due to the diffusion of saponin.

(4) It is suggested that the slow changes which occur in the tension of certain plastic bodies following a strain may also be due to the diffusion of molecules into positions where they affect the tension.

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Embryo-sac Formation in Diploid and Polyploid Species of Roseæ.

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[PLATES 10–15.]

Introductory Note.

During the past ten years, the author has been carrying out cytogenetical experiments with the numerous species of the Tribe Roseæ of the Family Rosaceæ. In the course of this research, 691 varieties have been examined cytologically and taxonomically, including representatives of 198 Linnean species and many natural and genetical hybrids of the six genera of the Tribe. Several workers have investigated the processes of pollen formation and male gametogenesis, but, owing to various technical difficulties, no one has yet published a consecutive and comprehensive account of the processes of embryo-sac formation in the species of this Tribe. Hoffmeister (1858) and Strasburger (1878) examined a few species of *Rosa* and found that several embryo-sacs were formed in one ovule. Péchoutre (1902) discovered two fully-developed embryo-sacs in one ovule in one species of *Rosa*, and both he and Strasburger found that the micropylar cell of the quartet develops to form the embryo-sac. Täckholm (1920, 1922) examined the formation of embryo-sac mother cells in the irregular polyploid species of the *Caninæ* Section of the genus *Rosa*, and discovered the remarkable unequal reduction division that takes place in the embryo-sac mother cells of these species, but technical difficulties in sectioning the older ovules and achenes prevented an investigation of the later stages. It seems desirable, therefore, to publish a general account of the processes of embryo-sac formation in Roseæ, since the research covers new ground.

The various stages of female gametogenesis have been investigated in 193 varieties of 104 Linnean species of the Tribe, including the diploid species of *Rosa* L., *Platyrhodon* Decn., and *Hesperhodos* Ckll., and the regular and irregular polyploid species of *Rosa* L. Many crosses and hybrids have also been studied in this respect, but owing to their peculiar behaviour it is necessary, for a clear account, to give comparative pictures of both male and female gametogeneses, so that they cannot be included in this report. The outstanding cytological feature of the species of the Tribe Roseæ is the constancy of their basic number of chromosomes. With the exception of a few hybrids, all the 1130 individuals so far examined by seven workers in Europe and America are Euploid, with 7 as the basic number. Of the six genera composing the Tribe, all the species are diploid, except those of *Rosa* L., in which the prevalence of polyploidy is a remarkable feature. In this genus there are diploids with 14 somatic chromosomes, triploids with 21, tetraploids with 28, pentaploids with 35, hexaploids with 42, and octoploids with 56 somatic chromosomes. Of these, most of the tetraploid and hexaploid species, and all the examined octoploid species are quite regular in the formation of their male and female gametes. The triploids are all of hybrid or garden origin, and no established triploid species has so far been found in Nature. The remaining tetraploid and hexaploid species and all the pentaploid species, which together comprise the old Section *Caninæ* (*sensu latissimo*), *i.e.*, *Caninece*, Christ, 1873, are all irregular in their cytological behaviour and are distinguished by a peculiar mechanism of gametogenesis which is at present unique in plants and animals. In these irregular polyploid species the functional male gametes have only one septet of 7 chromosomes, the remaining septets- 3 in tetraploids (21), 4 in pentaploids (28), and 5 in hexaploids (35)—being lost in the processes of gametogenesis. The male heritage of all these irregular polyploid species is therefore that of a simple diploid species.

On the other hand, the female reduction division in these species is regular but unequal, producing female gametes with 3 septets of chromosomes (21) in the tetraploid, 4 septets (28) in the pentaploid, and 5 septets (35) in the hexaploid species; 1 septet of chromosomes (7) being lost in each case. The female heritage of these irregular polyploid species is consequently that of a regular polyploid species higher than itself, *i.e.*, an irregular tetraploid produces the female gametes of a regular hexaploid, a pentaploid those of an octoploid, and an irregular hexaploid those of a regular decaploid species.

By means of this remarkable mechanism, the specific somatic number of chromosomes is maintained whenever sexual reproduction occurs, notwithstanding the inequality of the reduction divisions.

These irregular polyploid species of *Rosa* are, however, only facultatively sexual in their reproduction, since their seeds are for the most part apomictical, reproducing the mother parent in every detail. Since the frequent crosses and hybrids produced by sexual reproduction are truly perpetuated from the first by apomictical reproduction, these species are extremely polymorphic in their characters.

Judging by the fossil remains of these species, this double process of hybridisation and apomixis has been going on from the time that they first appeared as hybrids or chromosomal transmutations of the regular polyploid and diploid species on the retreat of the ice of the Great Mindel glaciation in the Pleistocene Period. The present geographical distribution of these species is confined to Europe, Western Asia and North Africa, over an area which broadly corresponds with the area influenced by the Great Mindel glaciation in its advance and retreat.

Material and Methods.

Material.—Flower buds were collected from wild bushes growing in the Midland and Eastern Counties of England and in North Wales; in Switzerland, in the Upper and Lower Engadine, in the Cantons Uri, Ticino and Schwyz, and in the northern foothills of the Jura Mountains by the author and his wife: in Siberia, around Lake Baikal and in Turkestan, by Professor T. D. A. Cockerell, of Colorado, U.S.A. Collections were also made in the Royal Botanic Gardens, Kew, in the Botanic Gardens of Oxford, Cambridge, and Basel; in the collections of Colonel Gravereaux at La Belle Roseraie de l'Haÿ, Paris, of the late Canon Carew-Hunt at Albury, Oxford, and of the author at Burbage, Leicestershire (now at Cambridge). Many interesting results were obtained from plants grown from seeds collected for me by Professor Heslop Harrison, F.R.S., in Co. Durham, Professor Stanley Gardiner, F.R.S., in France, Professor Cockerell in America and Asia, Dr. Eileen Erlanson in America, the late Dr. Gadow, F.R.S., in Mexico, Dr. Hugh Scott in Abyssinia, and several correspondents in Canada and Japan. I am much indebted to Professor A. C. Seward, F.R.S., and Professor Stanley Gardiner, F.R.S., for laboratory accommodation at Cambridge, and to Mr. F. T. Brooks, F.R.S., for his personal instruction and early supervision in cytological technique. My wife has given me much assistance in the microscopical preparations and has made all the camera lucida drawings. I am especially indebted to the generosity of the Government Grant Committee of the Royal Society for a grant in aid of these experiments and researches, which, without their assistance, would have come to an untimely end.

Methods.—For the purpose of investigating embryo-sac formation, Carnoy's Fluid was found to be the most satisfactory fixative, since its rapid fixing and greater powers of penetration ensured good preparations, which were not easily achieved by other methods when dealing with the hard bony achenes of *Rosa*. In the course of the work it was found advantageous to leave the material in Carnoy's Fluid for a considerable period, at least 24 hours and preferably longer, even up to 3 or 4 weeks, since material treated in this way showed better fixation and good penetration of the paraffin wax, which consequently led to easy sectioning.

Various stains were used, according to the stage to be investigated. Iron-Alum—Hæmatoxylin gave good pictures of embryo-sacs, but for counts of chromosomes Gentian-Violet—Orange G was found to be a more satisfactory stain. In other cases Flemming's Triple Stain, Cyanin-Erythrosin and other transparent stains were used.

In examining the microscopical preparations, a Watson's Oil-Immersion Condenser was used (aperture 1·34) with either a 1·5-mm. Zeiss apochromatic objective or an apochromatic $\frac{1}{12}$ with ×10 eye-piece for searching and ×20 for detailed examination.

All the drawings were made with an Abbé camera lucida with the board at table level.

Embryo-sac Formation in Diploid and Regular Polyploid Species of Roseæ.

The processes of megaspore formation are so similar throughout all the regular species, whether diploid or polyploid, that one description will cover them all. The first differentiation of the megasporangial tissue takes place immediately before or during the first division of the pollen mother cells. The integuments appear at this time and grow rapidly, the nucellar tissue quickly adding layer after layer. During this growth the embryo-sac mother cells, of which many form in each ovule, become elongated and pass into synizesis about the time of the reduction division in the pollen mother cells or rather later. This condition persists for a considerable time, and while the pollen mother cells complete their second division and while the resulting microspores grow into pollen grains, the size of the ovule rapidly increases. The integuments grow up and join at the top of the nucellus and many more layers are added to the latter. The embryo-sac mother cells become much elongated and form a nest of long and rather narrow cells in the lower part of the ovule. The number found in each ovule is variable even in the same flower-bud, but there are commonly about four layers at the chalazal end each with four cells and frequently one or two apical layers above. A few species, notably the CC diploid septet species *R. rugosa* Thunb. em., have comparatively few embryo-sac mother cells, occasionally only two or three, but usually eight or nine.

The nuclei pass through the same stages as the p.m.c. and the reduction division (fig. 2, Plate 10) usually coincides with the first division of the nuclei in the young pollen grains. Cytomixis has not yet been observed in the e.m.c., and as it apparently depends much upon external conditions, it is possible that the embryo-sac mother cells being in a more protected position than the anthers are less likely to be affected. During the division stages, the presence of many e.m.c. is a great advantage since one may find divisions going on for a considerable period and all stages may be found, from synizesis to the completed quartet, in one and the same ovule. The inner cells are the first to divide and the early stages and first division are similar in all respects to the corresponding division in the p.m.c., the chromosomes forming typical gemini, 7 in the case of the diploid species ($2n = 14$), 14 in the tetraploid ($2n = 28$), 21 in the hexaploid ($2n = 42$) (fig. 1, Plate 10), and 28 in the octoploid species ($2n = 56$). The anaphase and telophase stages are carried through with great regularity and a dyad is formed with a cell plate dividing it into two cells (fig. 3, Plate 10). The second division spindles are formed in the same plane as the first division spindle, one above the other with the poles pointing to the micropylar and chalazal ends of the ovule respectively (figs. 2 and 4, Plate 10). Thus a regular quartet is formed, the four cells lying in a more or less straight row (fig. 5, Plate 11).

One remarkable case of irregularity was observed in a tetraploid (labelled *R. Beggeriana*, but more nearly allied to *R. Fedschenkoana* Regel), found at Kew. The p.m.c. divisions showed an orderly and regular tetraploid behaviour with 14 gemini and regular divisions. Since the plant was observed to be sterile, the e.m.c. were examined and in most cases there were 28 single chromosomes at diakinesis instead of the 14 gemini, or more rarely a variable number of gemini and singles. The singles appeared to reduce irregularly at the first division, and did not carry through an equatorial division, causing great irregularity and rapid degeneration. The presence of occasional embryo-sacs may have been the result of more complete pairing and consequently regular reduction or by the equatorial division of a 28 single chromosomed nucleus giving a "diploid" embryo-sac. The presence of apomixis consequent on this irregular behaviour is, however, unlikely since the plant was almost infertile and the few seeds sown failed to germinate. It is possible that the plant is a hybrid though, if so, the regularity of the pollen divisions is remarkable. This was the only case of irregularity found in the e.m.c. of species examined, all others behaved normally.

In all the regular species, while the innermost cells, which have divided first, are preparing for further development, the outer cells carry out their division. In some cases, degeneration sets in quite early in these outer cells giving rise to irregularities of division such as lagging chromosomes, so that it is not safe to take the behaviour of the chromosomes of these cells to be typical for the plant. In most cases, however, they perform a regular reduction and many reach a 2-nucleate embryo-sac stage or even attain to the 4-nucleate stage. Their continuance or discontinuance, however, depends largely on the number of e.m.c. present, those ovules with many showing much earlier degeneration of the outer cells than those with few. There is also a great difference in the stage of development between the ovules themselves. In the *AA* and EE septet species and some sub-species of the BB septet species, in which the achenes are basi-parietal, the ovules in those at the base of the receptacle are much more advanced than those up the sides, while in the CC and DD septet species and other sub-species of the BB septet species, in which the insertion is mostly basal, the ovules are all at approximately the same stage. In some cases, the outer cells remain in synizesis for a long time and one may often see some in this condition at quite a late stage of embryo-sac development. As a rule, however, they degenerate fairly early and are absorbed by the rapidly growing central sacs.

The central megaspores which reduced first are now developing into embryo-sacs. The micropylar cell of the quartet usually enlarges to form the functional embryo-sac (fig. 6, Plate 11), but this is not entirely constant, for one may occasionally see the chalazal (fig. 5) or even one of the two central cells continuing in some cases. This condition does not appear to be confined to any particular species since one may see the two conditions in different ovules in the same flower-bud or even in different quartets of the same ovule in some species. Afzelius (1924) found a similar condition in certain species of *Senecio* and allied genera. In these genera the chalazal cell usually persists, but in some cases the micropylar or one of the middle cells of the quartet appeared to be enlarging for division. Afzelius doubts, however, whether these would-continue and he thinks it is possible that they would degenerate later and their place be taken by the smaller chalazal cell. It is difficult to follow the later stages of the quartet in *Rosa* in order to determine the ultimate fate of the cells or to judge the previous position of the developing one with regard to the others. The three cells which do not continue, degenerate with great rapidity and their places are quickly taken by the various embryo-sacs which are in course of formation, all trace of their former position being obliterated and the whole mass of growing embryo-sacs and still undivided lateral cells form such a confused assembly that it is almost impossible to sort them out with any degree of accuracy until a later stage, save in the few cases in which only one or two sacs are formed.

The first sign of embryo-sac formation is the enlargement of the cell, which is to continue, often accompanied by vacuolation of the cytoplasm. The nucleus also enlarges considerably and may often be pushed towards the side of the cell by a large vacuole, giving it a very similar appearance to the pollen grain just before the first division, as it appears in many species. The division takes place on the same plane as the previous divisions and the two nuclei resulting lie each at one end of the now rapidly elongating sac, usually separated by a large vacuole (fig. 7, Plate 11). The second division (figs. 8, 9 and 10, Plate 11) occurs quickly, and as a rule after the division there may be seen two nuclei lying closely together at each end of a long and narrow sac separated by a long narrow vacuole (fig. 11, Plate 11). In a few cases, in which the sac is broader and less elongated, the four nuclei lie in a quartet-like row in a short, thick embryo-sac (fig. 12, Plate 12).

The sac now enlarges considerably and penetrates farther and farther up towards the micropyle until finally it reaches as far as the junction of the integuments. Usually it ceases here, but sometimes it may penetrate nearly through the integuments or, curling round half-way, grow up the space between the nucellus and the integuments for the last part of the way. The last division to the 8-nucleate stage (fig. 13, Plate 12) does not take place until very late, in many cases not until the pollen tubes are already half-way down the styles, but more generally when the pollen is just beginning to germinate on the stigma. This last division takes place very rapidly, the cytoplasm surrounding the nuclei furrows and two synergids form at the extreme apex of the sac with the egg cell immediately below, while the first polar nucleus moves quickly down the long narrow sac to meet the second polar nucleus (fig. 16, Plate 12). The meeting takes place about one-third of the way down the sac (fig. 17, Plate 12) except in cases where division of the lower cells has been somewhat belated, when it may take place lower down. The two nuclei lie closely pressed together, but do not fuse before fertilisation. The three antipodal nuclei quickly disappear. They sometimes lie in an axial row (fig. 15, Plate 12), but more usually form a triangle with the apex to the chalazal end of the sac (fig. 14, Plate 12). The cytoplasm furrows and three small cells are formed, which rapidly degenerate.

Meanwhile the other e.m.c. also have been dividing and developing to form embryo-sacs. In some cases a second cell has kept pace with the first, giving two fully developed sacs, but frequently the other sacs formed (usually from two to six) remain at the 4-nucleate stage and degenerate about the time of fertilisation. When two 8-nucleate sacs occur, one is usually rather above the other, often curled over it, and hence is fertilised first (fig. 20, Plate 13), the second sac rapidly degenerating. No cases have been seen of two sacs being fertilised in the same ovule, and only very rarely do more than two 8-nucleate sacs develop in the regular species.

In spite of the large amount of material cut, the actual stage of fertilisation has been missed, although pollen tubes have been seen penetrating the micropyle (fig. 18, Plate 12) and the immediate after-stages have also been observed. Apparently fertilisation does not take place until several days after the fading of the flower. After fertilisation the fertilised egg cell remains dormant, while the endosperm nuclei begin to divide. When a single lining of free endosperm nuclei has been formed around the sac, the first division of the fertilised egg cell takes place (fig. 19, Plate 13) and a normal embryo quickly develops (figs. 21, 22, 24, Plate 13). The endosperm now commences cell formation, beginning first in the centre and top of the sac and forming a layer of very thin hexagonal cells (fig. 23, Plate 13). Later it grows inwards and more or less fills up the cavity of the sac, but disappears rapidly as the young embryo grows and fills up the sac, until in the end only a single layer is left.

Endosperm divisions observed in *R. Sayi*, Schwein, a hexaploid species ($n = 21$, $2n = 42$), showed that triple fusion had taken place. The chromosomes were very thin and elongated, which made counting difficult, but it was obvious that the number was about 60, and a few good plates gave clear counts of 63 (fig. 25, Plate 13).

Seminal reproduction in the diploid and regular polyploid species of *Rosa* is apparently wholly sexual, and there is no genetical or cytological evidence of apomixis in these species.

Table I.—Diploid and Regular Polyploid Species.

List of Diploid and Polyploid Species of the Tribe *Roseæ* in which the Embryo-sacs have been examined by the author, with the number of Varieties examined in each Species and the number of Male and Female Gametic Chromosomes and Somatic Chromosomes found. The Species are arranged in their Cytogenetical Groups with Septet and Linnean Species (Hurst, 1928).

Vars.	Groups.	Chromosomes.	
		Gametic.	Somatic.
	TRIBE *Roseæ*.		
	Genus *Rosa*. L.		
88	DIPLOID SPECIES—	*7*	*14*
21	AA Septet Species—	*7*	*14*
3	*R. arvensis*, Huds.	7	14
1	*R. moschata*, Herrm.	7	14
1	*R. Brunonii*, Lindl.	7	14
1	*R. Leschenaultiana*, Wight et Arn.	7	14
2	*R. longicuspis*, Bertol.	7	14
1	*R. Soulieana*, Crép.	7	14
1	*R. Helenæ*, Rehdr. et Wils.	7	14
1	*R. Rubus*, Lév. et Van.	7	14
1	*R. Banksiæ*, Ait.	7	14
2	*R. chinensis*, Jacq.	7	14
1	*R. anemoneflora*, Fort. ex Lindl.	7	14
2	*R. multiflora*, Thunb.	7	14
1	*R. Wichuraiana*, Crép.	7	14
1	*R. setigera*, Michx.	7	14
2	*R.* (Linnean species, undescribed)	7	14
22	BB Septet Species—	*7*	*14*
3	*R. sericea*, Lindl. (excl. fig.)	7	14
6	*R. omeiensis*, Rolfe	7	14
2	*R. Webbiana*, Wall.	7	14
2	*R. sertata*, Rolfe	7	14
1	*R. Willmottiæ*, Hemsl.	7	14
1	*R. xanthina*, Lindl.	7	14
3	*R. Hugonis*, Hemsl.	7	14
2	*R. cabulica*, Boiss.	7	14
2	*R. gymnocarpa*, Nutt.	7	14
14	CC Septet Species—	7	*14*
5	*R. rugosa*, Thunb.	7	14
1	*R. nipponensis*, Crép.	7	14
4	*R. nitida*, Willd.	7	14
4	*R.* (Linnean species, undescribed)	7	14
19	DD Septet Species—	*7*	*14*
2	*R. cinnamomea*, L. 1759 (non 1753)	7	14
3	*R. Beggeriana*, Crép. (p.p. non Schrenk)	7	14
1	*R. Marettii*, Lév.	7	14
1	*R. Woodsii*, Lindl.	7	14
2	*R. Fendleri*, Crép.	7	14

Table I—(continued).

Vars.	Groups.	Chromosomes.	
		Gametic.	Somatic.
	DIPLOID SPECIES—(contd.)—		
	DD Septet Species—(contd.)—		
1	*R. bidenticulata*, Rydb.	7	14
1	*R. Johnstonii*, Rydb.	7	14
2	*R. blanda*, Ait.	7	14
3	*R. palustris*, Marsh (*R. carolina*, L. 1762 non 1753)	7	14
3	*R.* (Linnean species, undescribed)	7	14
12	EE Septet Species—	*7*	*14*
1	*R. macrophylla*, Lindl.	7	14
3	*R. corymbulosa*, Rolfe	7	14
1	*R. Giraldii*, Crép.	7	14
1	*R. elegantula*, Rolfe	7	14
1	*R. persetosa*, Rolfe	7	14
5	*R.* (Linnean species, undescribed)	7	14
61	REGULAR POLYPLOID SPECIES.		
47	REGULAR TETRAPLOID SPECIES—	14	28
11	AACC Septet Species— ..	*14*	*28*
6	*R. centifolia*, L.	14	28
3	*R. rubra*, Blackw. 1757 (*R. gallica* L. 1759 non 1753)	14	28
2	*R. damascena*, Blackw.	14	28
1	AAEE Septet Species— ..	*14*	*28*
1	*R. Davidii*, Crép. (*R. setipoda*, Hemsl. et Wils.)	14	28
6	BBCC Septet Species—	*14*	*28*
4	*R. spinosissima*, L. 1762	14	28
1	*R. myriacantha*, D.C.	14	28
1	*R.* (Linnean species, undescribed)	14	28
10	BBDD Septet Species—	*14*	*28*
1	*R. pimpinellifolia*, L. 1759	14	28
5	*R. altaica*, Willd.	14	28
1	*R. grandiflora*, Lindl.	14	28
1	*R. Fedtschenkoana*, Regel.	14	28
2	*R.* (Linnean species, undescribed)	14	28
1	CCDD Septet Species—	*14*	*28*
1	*R. virginiana*, Mill. (*R. lucida*, Ehr.)	14	28
18	DDEE Septet Species—	*14*	*28*
14	*R. pendulina*, L. 1753 (*R. alpina*, L. 1762)	14	28
1	*R. Hawrana*, Kmet ex Kern.	14	28
1	*R. laxa*, Retz. in Hoffm. (non Lindl., nec Hort.)	14	28
2	*R.* (Linnean species, undescribed)	14	28
11	REGULAR HEXAPLOID SPECIES ..	*21*	*42*
4	AABBEE Septet Species—	*21*	*42*
2	*R. Moyesii*, Hemsl. et Wils.	21	42
2	*R. Sweginzowii*, Koehne in Fedde ...	21	42

Table I—(continued).

Vars.	Groups.	Chromosomes.	
		Gametic.	Somatic.
	REGULAR HEXAPLOID SPECIES—(contd.)—		
1	AACCEE Septet Species—	*21*	*42*
1	*R. Hemsleyana*, Tackh.	21	42
4	AADDEE Septet Species—	*21*	*42*
3	*R. nutkana*, Presl.	21	42
1	*R. Nuttalliana*, Hort. Kew	21	42
1	BBDDEE Septet Species—	*21*	*42*
1	*R. Engelmanii*, S. Wats.	21	42
1	CCDDEE Septet Species—	*21*	*42*
1	*R. Sayi*, Schwein.	21	42
3	REGULAR OCTOPLOID SPECIES— ..	*28*	*56*
2	AACCDDEE Septet Species—	*28*	*56*
1	*R. acicularis*, Hort. Kew (*R. acicularis*, Lindl. p.p. cum icon.)	28	56
1	*R. acicularis*, Hort. Gravereaux	28	56
1	BBCCDDEE Septet Species— .	*28*	*56*
1	*R. baicalensis*, Turcz. .. .. (*R. acicularis*, Lindl. p.p. excl. fig.) (*R. acicularis*, Hort. Cantab.)	28	56
	Genus *Platyrhodon*, Decaisne.		
1	*Platyrhodon microphylla*, Decn..... . (*Rosa microphylla*, Roxb. ex Lindl.) (non Desf.) (*Rosa Roxburghii*, Tratt.) (*Saintpierrea microphylla*, Germain)	7	14
	Genus *Hesperhodos*, Cockerell.		
1	*Hesperhodos minutifolia*, subsp. *mirifica*, Hurst (*Rosa mirifica*, Greene) (*Hesperhodos mirifica*, Ckll.)	7	14

Embryo-sac Formation in the Irregular Polyploid Species of ROSA *L.*

The formation of the female gametes has been examined in a representative set of the Irregular Tetraploid and Pentaploid species of *Rosa L.*, and the discovery of the unequal reduction division in the embryo-sac mother cells by Tackholm (1920 and 1922) has been fully confirmed. A large number of embryo-sac mother cells is formed in most cases, several more than those usually found in the regular species. Up to the reduction division the behaviour is the same as in the pollen mother cells, but after this it is entirely different. As in the regular species, the first meiotic division coincides approximately with the first division of the pollen grain nuclei. In diakinesis seven bivalent chromosomes appear, the chromosomes of the remaining septets being unpaired. In the first meiotic division the seven bivalents form a regular equatorial plate, but the univalents do not form up around them as in the pollen. As the bivalents pass to the plate the univalents all collect at the micropylar end of the cell (figs. 26 and 28, Plate 14), where they are presently joined by seven of the reduced bivalents which have carried through a normal reduction division (fig. 29, Plate 14). Thus a regular dyad is formed, the lower cell containing a nucleus with *only* seven chromosomes, while the upper one contains the other seven plus all the univalent chromosomes (figs. 27 and 30, Plate 14), giving the unequal but regular reduction division of 7–21 chromosomes in the tetraploid species, 7–28 in the pentaploids, and 7–35 chromosomes in the hexaploid species.

The second meiotic division is normal and regular, being equational and non-reductional as in the regular species except for the different number of chromosomes included on the two spindles (fig. 30, Plate 14), the lower one having only 7 while the upper spindle has 21, 28, or 35 respectively in tetraploids, pentaploids and hexaploids. A regular, but unequal quartet of megaspores is formed, the two upper micropylar cells and nuclei being much larger than the two lower chalazal cells and nuclei (figs. 32, 33 and 34, Plate 15). In this way a remarkable mechanism is established whereby, through unequal syngamy, the somatic number of chromosomes is carried on to the next generation. The upper micropylar cell of the quartet, with its complement of all the univalent chromosomes 14, 21 or 28, plus 7 of the bivalents, continues to form the embryo-sac and, being fertilised by a male gamete containing 7 chromosomes, re-establishes the original somatic number of 28 in the tetraploids, 35 in the pentaploids and 42 in the hexaploids.

Some deviations from this regular division occur, some univalents being seen at the chalazal pole and odd chromosomes are extruded from the daughter nuclei, but since these irregularities occur as a rule only in the outer cells which soon degenerate they have no more significance than similar irregularities in the outer cells of the regular species (pp. 130–131).

So far as observed, the central or inner cells which form the embryo-sacs were entirely regular in their unequal reduction. Tackholm (1922) found that about 75 per cent. of the embryo-sac mother cells carry through the regular unequal division while the author found 72 per cent., which is near enough to Tackholm's figure to confirm his statement.

These described divisions being reductional are obviously sexual. Although careful search has been made through a large amount of material there is so far little cytological evidence for the apomictical development of embryo-sacs which genetical experiments and data demand. Hundreds of diakineses observed all show the 7 paired chromosomes instead of all single chromosomes as would be expected in apomixis. Three cases have been seen, however, of metaphases in which all the chromosomes were on the equatorial plate (fig. 31, Plate 14) and appeared to be carrying through an equational, non-reductional division, and a few isolated telophases have been observed which appeared to have approximately equal numbers of chromosomes at each pole. Large numbers of quartets examined show two large micropylar cells and two small chalazal cells, but here also there are occasional quartets with approximately equal cells. If the equational divisions observed in the embryo-sac mother cells are the origin of the apomictical embryo-sacs there is a possibility that they may be formed straightway without passing through the sexual dyad and quartet stages, thus reducing the probabilities of observation. In the heterogeneous collection of early embryo-sac development with dyad and quartet formation in both the inner and outer and the upper and lower cells, it is extremely difficult to make accurate observations at this stage in order to determine which are single cells and which parts of dyads, quartets or embryo-sacs, especially as they may be often, not only curled round one another in the struggle for development, but also actually one above the other, giving a row of cells which may contain a single cell, a dyad and a quartet. As in the regular species, many of the outer cells remain in synizesis until a late stage, but these have no significance since later evidence shows that it is the first formed embryo-sacs that produce the embryos so that the apomictical divisions must be carried through first of all and also in the central cells which always produce the surviving embryo-sacs.

In the formation of the sexual embryo-sacs the topmost or upper micropylar cell of the quartet which carries all the univalents and the reduced number of bivalents, is usually the functional one, though in many cases the lower micropylar cell may divide once and occasionally even twice (figs. 34, 35, 36, Plate 15). The two chalazal cells, containing only the 7 chromosomes, usually degenerate rapidly, though even here one may at times find one division taking place (fig 36). There is the possibility that a fusion might take place between one of the micropylar nuclei and one of the chalazal, thus producing an apomictical sac. That the fusion should take place between adjacent quartets is excluded, since segregation would result, unless a perfect state of homozygosity is present in these species, in which case normal self-fertilisation would have the same result. If one of the lower cells should persist and form an embryo-sac an interesting result might follow in the formation of a diploid species by fertilisation with a 7 chromosome male gamete. Such an occurrence is remote, however, since the small sacs would be rapidly displaced by the larger ones, unless by some abnormality only chalazal cells continued. The subsequent development of the embryo-sacs is the same as in the regular species except that in the irregular species there are consistently two equal-sized and equally well-formed 8-nucleate sacs in each ovule, and often more, as many as five having been counted in one ovule. Many 4-nucleate sacs occur around the main ones as in the regular species (fig. 38, Plate 15), which eventually degenerate. The fact that at least two complete embryo-sacs are present in each ovule may be significant, since one may be sexual and the other apomictical as in *Hieracium* (Rosenberg, 1906), though in the case of *Hieracium* the apomictical sacs are of nucellar origin. Various counts taken in the developing embryo-sacs of *Rosa* all show the reduced number of chromosomes and, so far, none with the full somatic number have been observed. The occurrence of nucellar or integumental embryo-sacs with the somatic number of chromosomes as found by Rosenberg (1906) in *Hieracium*, and by Chiarugi (1926, 1927) in *Artemisia*, has not been observed in *Rosa*, in which the most probable source of apomictical embryo-sacs appears to be the equational divisions of embryo-sac mother cells, leading straightway to the formation of apomictical embryo-sacs with the full somatic number of chromosomes, the normal first reduction division being omitted. Later cuts in several species show in all cases a perfectly normal embryo (fig. 39, Plate 15) in one of the embryo-sacs, the others degenerating after persisting for a time. These embryos are apparently formed from an egg-cell in the usual way and are surrounded by endosperm as in the regular species. Divisions in the endosperm are difficult to find, and although a great deal of material has been cut, no divisions showing that triple fusion has taken place have been met with, although the adhesion of the two polar nuclei has been observed (fig. 38). If the embryo-sac is apomictical with a double fusion of polar nuclei instead of the sexual triple fusion, there should be 7 more chromosomes present in the endosperm than in a fertilised one, since the male gamete would only bring in

7 chromosomes while the two apomictical polar nuclei would each have 7 chromosomes more than those in sexual sacs. (Apomictical double fusion, 35 + 35 = 70. Sexual triple fusion, 28 + 28 + 7 = 63). Since there is always a large percentage of bad pollen in these species, the pollen-grains on the stigmas are not so numerous as in the regular species, but a few grains may be observed germinating on the stigmas and their tubes penetrate freely down the styles. The actual stage of fertilisation has been missed, but in some cases the tubes have been seen entering the micropyle. In *R. glaucophylla var. jurassica*, collected by the author at Schuls in the Lower Engadine, a pollen-tube was seen approaching the embryo-sac. In view of the fact that hybrids are easily produced in crossing these irregular species with regular and other irregular species, and that the progeny show the expected number of chromosomes from a reduced sexual embryo-sac, it is evident that the sexual sacs persist, and it may be that the apomictical sacs are also present and function in the absence of fertilisation. That the own pollen of the irregular species should fail to function is remarkable, but the absence of any segregation or variation in "selfed" progenies shows that they must either be produced apomictically or that there is a definite and unusual homozygosity within the varieties of the species. Self-fertility in these species has not been demonstrated, and self-sterility leading to the functioning of the apomictical sacs in the absence of foreign pollen may be the explanation, since the pollen of the irregular species put on to other irregulars and on to regular species functions normally, though naturally not so freely as that of species which have 100 per cent. perfect pollen. In those species in which very young embryos were seen, and in a few cases where the endosperm was forming while the egg-cell still remained dormant, there was no apparent sign of fertilisation having taken place, such as degenerate synergids, pollen tubes and triple fusion, the sacs certainly having the appearance of continuing without fertilisation, although everything else was carried on apparently as normally as if fertilisation had actually taken place.

Table II.—Irregular Polyploid Species.

List of Irregular Polyploid Species of the Genus *Rosa* in which Embryo-sac Formation has been examined by the author, with the names of the varieties in each species and the number of male and female gametic chromosomes and the somatic chromosomes found in each. The species are arranged in their cytogenetical groups and septet species, each letter in the specific formulæ representing a septet of chromosomes and characters corresponding with the gametic septets of the five Differential Diploid Species of *Rosa* from which the Irregular Polyploid Species have been derived (*vide* Hurst, 1928). Provisional oldest names have been given to each Septet Species, but further research may lead to the discovery of still older legitimate names.

Groups.	Chromosomes.		
	Gametic.		Somatic.
	Male.	Female.	
Genus *Rosa*, L.			
Sectio Caninæ (*sensu latissimo*).			
AA SEPTET GROUP.			
Pentaploid Species.			
AABDE Septet Species—			
R. canina, L., 1753 em.			
,, ,, var. *lutetiana* (Lém.)	7	28	35
,, ,, var. *sphaerica* (Gren.)	7	28	35
,, ,, var. *nemophila* (Déségl. et Ozan.)	7	28	35
,, ,, var. *dumetorum* (Thuill.)	7	28	35
,, ,, var. *andegavensis* (Bast.)	7	28	35
AACDE Septet Species—			
R. mollissima, Willd., 1787 non Fries (*R. tomentosa*, Sm.)			
,, ,, var. *semitalis* (Rouy)	7	28	35
,, , var. *intromissa* (Crép.)	7	28	35
,, , var. *insidiosa* (Rouy)	7	28	35
,, ,, var. *alsatica* (Rouy)	7	28	35
,, ,, var. *lanuginosa* (Rouy)	7	28	35
Undetermined Septet Species—			
R. stylosa, Desv., 1809.*			
,, ,, var. *Desvauxiana*, Ser. in D.C.	7	28	35
,, .. var. *systyla* (Bast.)	7	28	35

* The septet formula of this peculiar species has not yet been determined. It produces an abundance of hips which usually contain only a few good seeds (1–3) and is confined for the most part to the lowlands of Western Europe. Déséglise placed the species in the section *Synstylæ* (whose species are all AA diploids). Christ placed it in the section *Caninæ* (which includes

Table II—(continued).

Groups.	Chromosomes.		
	Gametic.		Somatic.
	Male.	Female.	
AA SEPTET GROUP—(contd.)—			
Pentaploid Species—(contd.)—			
AABCD Septet Species—			
R. agrestis, Savi, 1798			
,, ,, var. *Bernardii* (Rouy)	7	28	35
,, ,, var. *sepium* (Thuill.)	7	28	35
× *R. bigeneris*, Duff. ex Rouy (*eglanteria* ♀ × *micrantha* ♂)	7	28	35
AABCE Septet Species—			
R. micrantha, Sm. 1800			
,, ,, var. *typica* (Christ)	7	28	35
,, ,, var. *microcarpa* (R. Kell.)	7	28	35
,, ,, var. *operta* (Puget)	7	28	35
BB SEPTET GROUP.			
Pentaploid Species.			
ABBCD Septet Species—			
R. eglanteria, L., 1753 (*R. rubiginosa*, L. 1771)			
,, ,, var. *rotundifolia* (Rau)	7	28	35
,, ,, var. *heteropoda* (Rouy)	7	28	35
,, ,, var. *apricorum* (Rip.)	7	28	35
,, ,, var. *comosa* (Rip.)	7	28	35
ABBCE Septet Species—			
R. elliptica, Tausch., 1819 (*R. graveolens*, Gren. et Godr.)			
,, ,, var. *typica* (Rouy)	7	28	35
Hexaploid Species.			
ABBCDE Septet Species—			
R. inodora, Fries, 1814 (non Borr. nec. auct. Britt.)			
,, ,, var. *typica*	7	35	42
CC SEPTET GROUP.			
Tetraploid Species—			
CCDE Septet Species—			
R. pomifera, Herrm., 1762 (*R. villosa*, L. p.p.)*			
,, ,, var. *Grenierii* (Déségl.)	7	21	28
,, ,, var. *recondita* (Puget)	7	21	28
,, ,, var. *murana* (Rouy)	7	21	28
,, ,, var. *arvernensis* (Rouy)	7	21	28
,, ,, var. *meridionalis* (Rouy)	7	21	28
,, ,, vra. *iserana* (Rouy)	7	21	28
,, ,, var. *rosgesiaca* (Rouy)	7	21	28

all the other pentaploid species), while Crépin created a new section for it called *Stylosæ* and suggested that it might be a hybrid between *R. arvensis*, Huds., of the *Synstylæ* and *R. canina*, L., of the *Caninæ*. Its taxonomic septet formula is approximately 4A + 1C + 1D + 1E, while cytologically it behaves as if it were 2A + 1C + 1D + 1E, having usually 7 bivalent and 21 univalent chromosomes in both p.m.c. and e.m.c. with 7 chromosomes in its male and 28 in its female gametes. That the bivalents are AA is evident from experimental genetical tests which show them to be pure and probably homozygous AA. The genetical tests of the remaining 3 septets, involving at least 4 independent hybridisations, are not yet completed.

* Formerly the tall, smooth, cany and almost prickleless forms of *R. pomifera*, often found in botanic gardens and in the lower regions of the Swiss Alps, were placed in the septet species CDEE (Hurst, 1928), but recent genetical experiments at Cambridge have demonstrated that these ecological forms more properly belong to the septet species CCDE and are not specifically distinct from the dwarfer and more prickly forms of the High Alps.

Table II—(continued).

Groups.	Chromosomes.		
	Gametic.		Somatic.
	Male.	Female.	
CC SEPTET GROUP—(contd.)—			
Pentaploid Species.			
ACCDE Septet Species—			
R. uriensis, Lagg. et Pug. ex Crép. 1869			
,, ,, var. *pubescens* (R. Kell.)	7	28	35
DD SEPTET GROUP.			
Tetraploid Species.			
CDDE Septet Species—			
R. mollis, Smith, 1812 (*R. mollissima*, Fries, non Willd.)*			
,, ,, ,, var. *typica* W-Dod	7	21	28
ADDE Septet Species—			
R. glauca, Pourret (non Vill.). (*R. rubrifolia*, Vill.)			
,, ,, var. *pseudo-glauca* (Rouy)	7	21	28
,, ,, var. *dispersa* (Rouy)	7	21	28
Pentaploid Species.			
ABDDE Septet Species—			
R. glaucophylla, Winch, 1816 (*R. glauca*, Vill.)			
,, ,, var. *jurassica* (Rouy)	7	28	35
,, ,, var. *platysepala* (Rouy)	7	28	35
,, ,, var. *falcata* (Rouy)	7	28	35
,, ,, var. *brevipes* (Rouy)	7	28	35
ACDDE Septet Species—			
R. caesia, Smith, 1811. (*R. coriifolia*, Fries)			
,, ,, var. *venosa* (Swartz)	7	28	35
ABCDD Septet Species—			
R. pseudo-mollis, Ley, 1907. (*R. tomentosa pseudo-mollis*, E. G. Baker)			
,, ,, var. *typica*	7	28	35
EE SEPTET GROUP.			
Pentaploid Species.			
ACDEE Septet Species—			
R. Froebelii, Christ ex Grav., 1902 (*R. laxa*, Hort. non Retz. nec Lindl.)			
,, ,, var. *laxa*	7	28	35

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* This northern and western species is not usually found in Britain south and east of Derby, and since only one colony has been found in the Cambridge district (Lime Pit Hill nr. Fulbourn) it is possible that seeds from the north have been transported by migratory birds. Incidentally this introduction of *R. mollis*, Sm., to Cambridgeshire has given rise to a new natural hybrid with the native *R. eglanteria*, L. 1753, with which it grows (*R. eglanteria* ♀ × *mollis* ♂) and well combines the characters of these two species. *R. eglanteria*, L. 1753, has the septet formula ABBCD, producing female gametes ABCD, while *R. mollis*, Sm., has the septet formula CDDE, producing male gametes D, the hybrid is therefore ABCDD with its female gametes identical with those of its mother parent *R. eglanteria* and its male gametes identical with those of its father parent *R. mollis*. In its characters and septet formula this natural hybrid is identical with the septet species *R. pseudo-mollis*, Ley, and provides a further illustration of the origin of a species by hybridisation, in this case in a new district (*cf.* × *R. bigeneris*, under *R. agrestis*, Savi). During the last eight years this natural hybrid has established *itself* as a species by apomictical seeds without variation.

Summary.

1. Embryo-sac formation has been investigated in 193 varieties of 104 Linnean species in 3 genera of the Tribe Roseæ.

2. A description is given of the various stages and processes of embryo-sac formation in the diploid and regular polyploid species of the Tribe.

3. The first and second meiotic divisions in these species are carried through in a regular and normal manner. The first division is the reduction division in which an equal number of chromosomes is segregated to each pole. The second division is equational, which leads to the formation of regular quartets arranged in an axial row. The micropylar cell of the quartet usually develops in to the normal 8-celled embryo-sac. Triple fusion takes place and after fertilisation normal endosperm is formed which, with the development of the embryo, is gradually reduced to a single layer. Minor variations occur from species to species and in some varieties, but the general mode of female meiosis and gametogenesis is normal and uniform throughout the diploid and regular polyploid species of the Tribe.

4. Throughout the Tribe several embryo-sacs are produced in each ovule, one of which ultimately survives in the regular species.

5. No evidence of the occurrence of apomixis was found in the diploid or regular polyploid species of *Rosa.*

6. A description is given of embryo-sac formation in the irregular polyploid species of the genus *Rosa* which is entirely different in mechanism and process from that of the diploid and regular polyploid species.

7. The reduction divisions of the embryo-sac mother cells are regular but unequal, giving rise to a mechanism of gametogenesis so far unparalleled in plants or animals.

This mechanism of female gametogenesis differs from that of the male gametogenesis in the same species as widely as both differ from the normal formation in the regular species.

In the first meiotic division, the 7 bivalent chromosomes alone reduce while the whole of the univalent chromosomes collect at the micropylar pole without splitting, thus giving rise to an unequal reduction division of chromosomes of 21 and 7 in the tetraploid species, 28 and 7 in the pentaploids and 35 and 7 in the hexaploid species.

The second meiotic division is equational and regular, resulting in the formation of unequal quartets placed in an axial row of two large micropylar and two small chalazal cells. The upper micropylar cell develops into an

8-celled embryo-sac, the egg-cells of which contain three, four or five times as many chromosomes as the male gamete.

8. The female heritage of these irregular polyploid species is consequently that of a regular polyploid species higher than itself, *i.e.*, an irregular tetraploid produces the female gametes of a regular hexaploid species, a pentaploid those of an octoploid and a hexaploid those of a decaploid species, while the male heritage of all these irregular polyploid species is that of a simple diploid species.

9. Experimental genetical evidence shows that these irregular polyploid species have an alternative method of reproduction by apomictical seeds. When "selfed" or agamised there is no varietal segregation, the progeny being completely identical with the mother parent. Self-fertility has not been demonstrated, but the species are faculatively sexual and hybridise freely when experimentally cross-fertilised with other varieties or other species.

10. Cytological evidence of this apomixis is scanty, although occasional divisions are found in the embryo-sac mother cells in which the reduction division is suppressed and these cells contain the full somatic number of chromosomes. So far, however, the few good counts available in embryo-sacs show only the reduced number of chromosomes.

11. The constant presence of two or more mature embryo-sacs in each ovule, peculiar to these species, may be significant, since one may be sexual and the other apomictical, the latter functioning in the absence of fertilisation.

12. Notwithstanding the large amount of material examined, no traces of the formation of embryo-sacs from nucellar or integumental tissue have been found in *Rosa*.

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DESCRIPTION OF PLATES.

Embryo-sac and Embryo Formation in Diploid and Regular Polyploid Species of Roseæ.

PLATE 10.

FIG. 1.—Diakinesis in the Hexaploid species *Rosa Moyesii* Hemsl. et Wils. (21 pairs of chromosomes).

FIG. 2.—Telophase of first meiotic division in the Diploid *R. gymnocarpa* Nutt. (7 pairs of chromosomes reduced).

FIG. 3.—Preparation for the second meiotic division in the Tetraploid *R. altaica*, Willd. (14 pairs of chromosomes reduced).

FIG. 4.—Second meiotic division in the Octoploid species *R. acicularis* Lindl. (p.p. cum icon.) with 28 chromosomes (the reduced number) in each cell.

PLATE 11.

FIG. 5.—Quartet in the Hexaploid species *R. nutkana* Presl in which the chalazal cell is enlarging to form the embryo-sac (rare in *Rosa*).

FIG. 6.—First division of the micropylar megaspore to form the embryo-sac in the Diploid *R. cabulica*, Boiss. The normal process in *Rosa*. (Telophase with 7 chromosomes at each pole.)

FIG. 7.—Two-nucleate embryo-sac in the Tetraploid species *R. centifolia*, L.

FIG. 8.—Second division in the embryo-sac of the Diploid *R. xanthina*, Lindl. (7 chromosomes).

FIG. 9.—Second division (chalazal nucleus) in the embryo-sac of the Tetraploid species *R. pendulina*, L.

FIG. 10.—Second division of the embryo-sac (7 chromosomes) in *Platyrhodon microphylla*, Decn.; on the right the same chromosomes more highly magnified.

FIG. 11.—Four-nucleate embryo-sac in the Diploid *Rosa pisocarpa*, A. Gray, showing usual appearance.

PLATE 12.

FIG. 12.—Second type of 4-nucleate embryo-sac, found occasionally, in which the nuclei lie in a row near to one another, in the Diploid *R. Hugonis*, Hemsl.

FIG. 13.—Third and last division in the embryo-sac of the Tetraploid *R. damascena*, Blackw., leading to the formation of 4 antipodal nuclei, each with 14 chromosomes (two cuts).

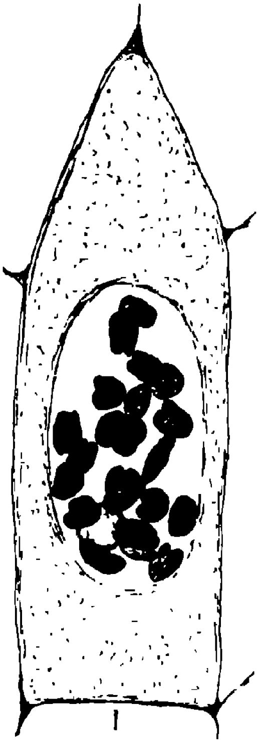
FIG. 14.—Cytoplasm furrowing round three antipodal nuclei to form three cells, in the Diploid species *R. rugosa*, Thunb.

FIG. 15.—Antipodal cells formed in a less usual manner, one above the other, in the Diploid *R. Hugonis*, Hemsl.

FIG. 16.—Eight-nucleate embryo-sac in the Diploid species *R. rugosa*, Thunb., polar nuclei approaching one another, egg-cell and two synergids above, three antipodal cells below.

FIG. 17.—Later stage in an 8-nucleate embryo-sac, the two polar nuclei lying pressed together, in the Diploid species *R. rugosa*, Thunb.

FIG. 18.—Pollen tube, with its male nuclei, penetrating the micropyle in the Diploid *R. nitida*, Willd.



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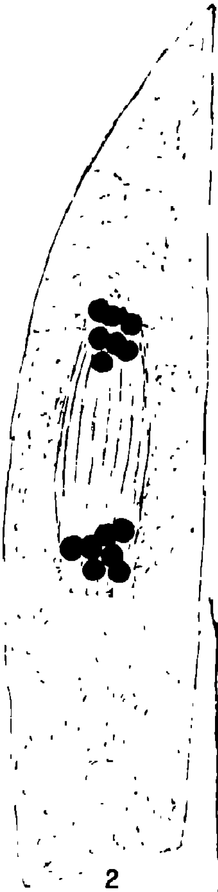

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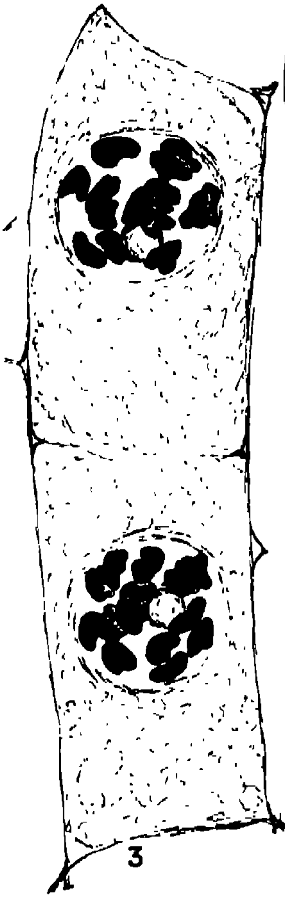

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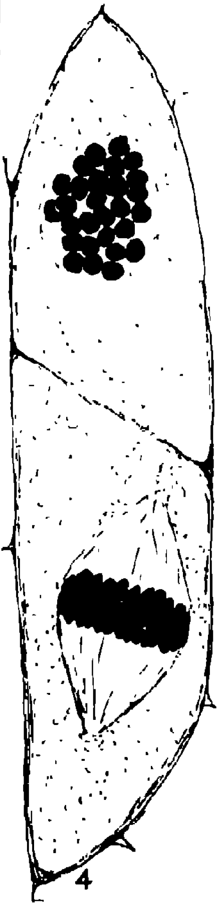

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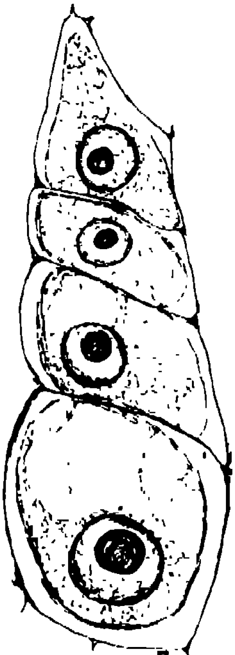
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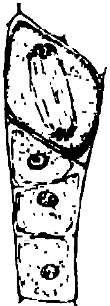
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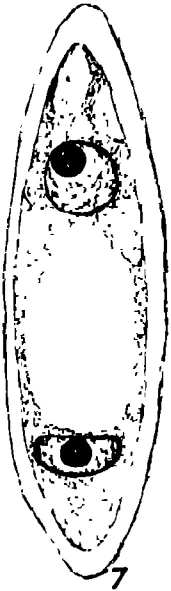
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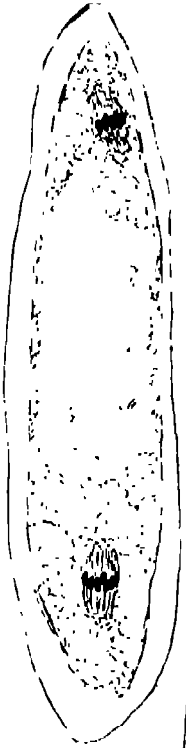
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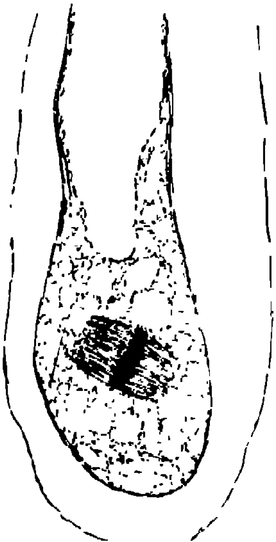
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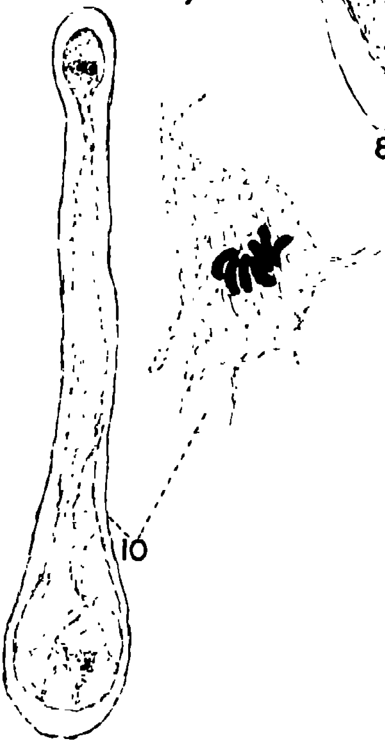
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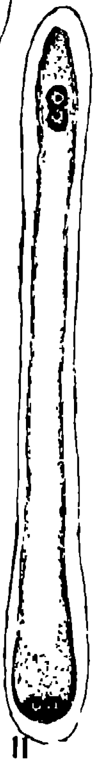
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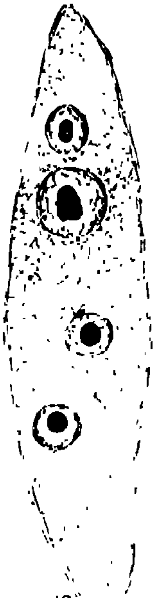

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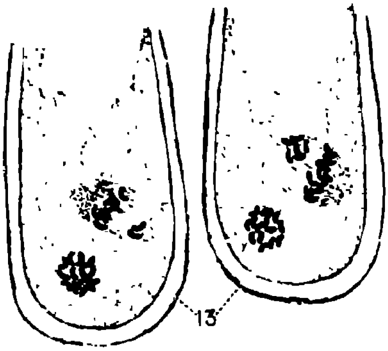

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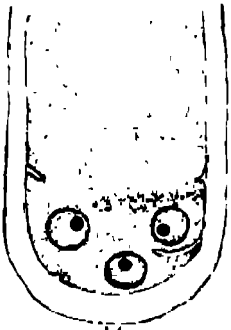

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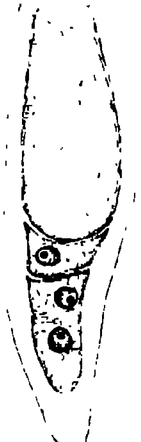

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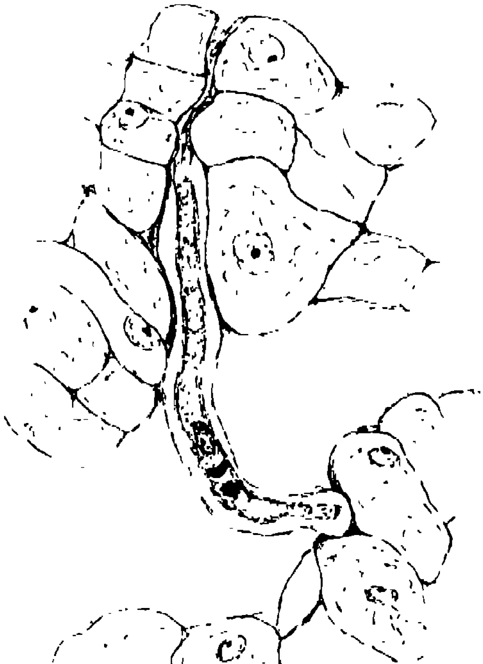

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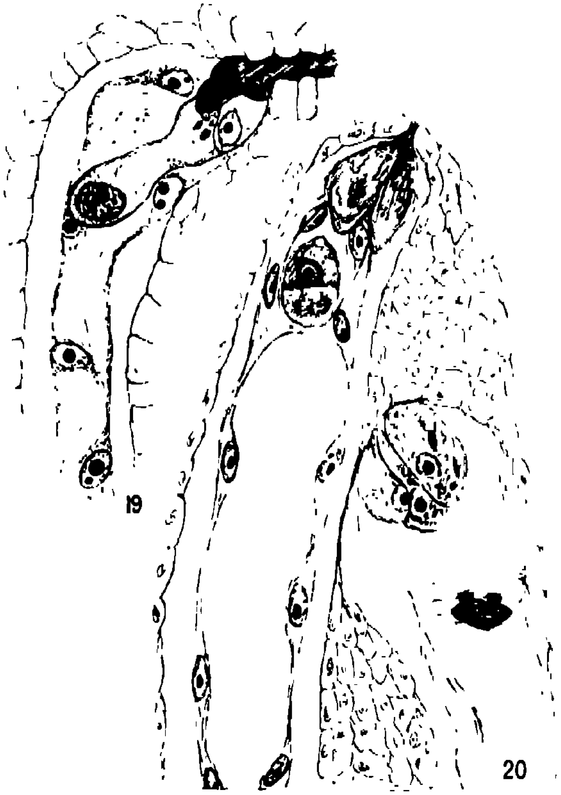

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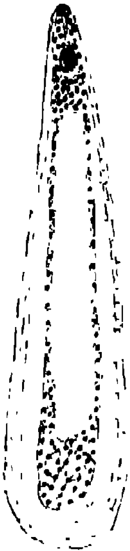

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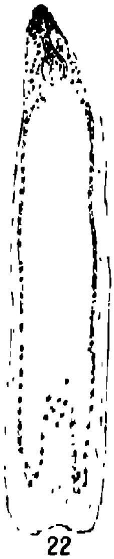

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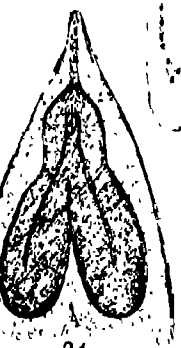

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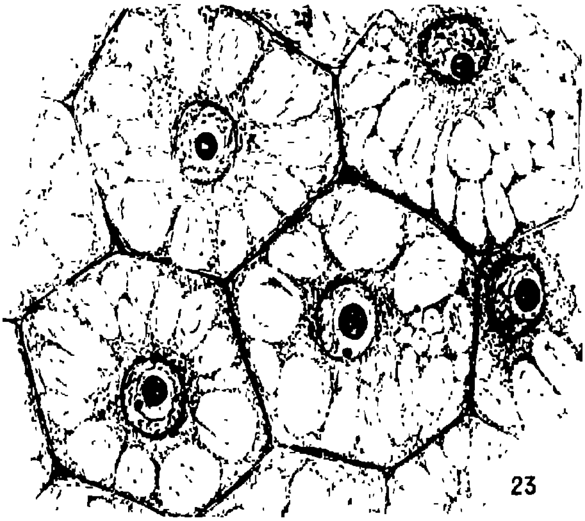

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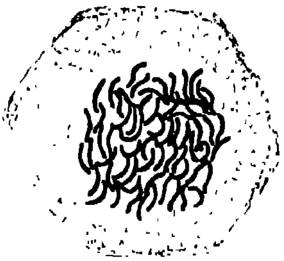

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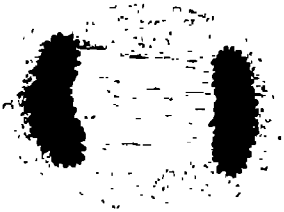

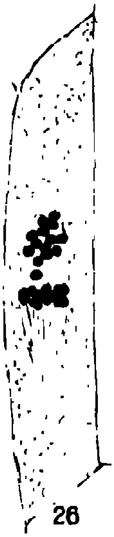

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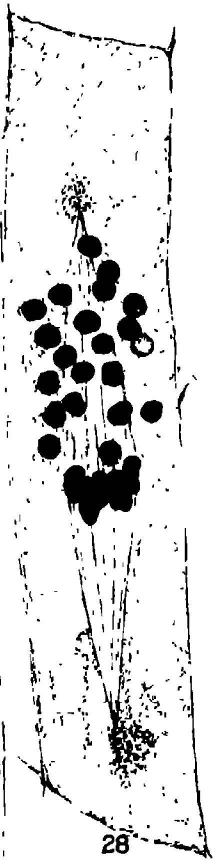

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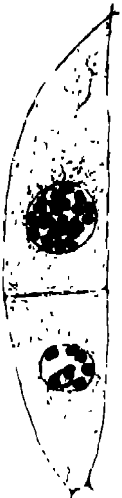

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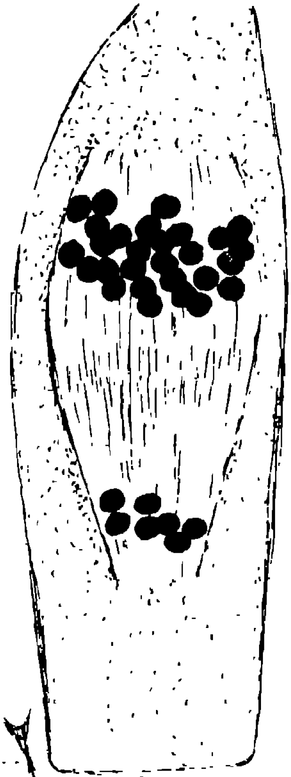

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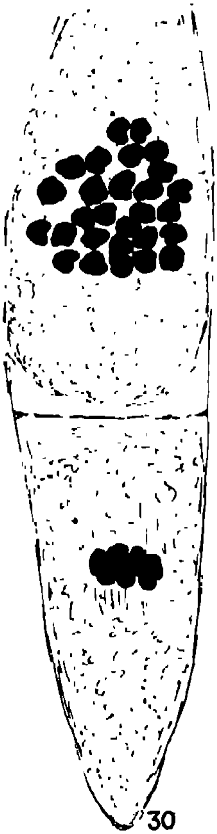

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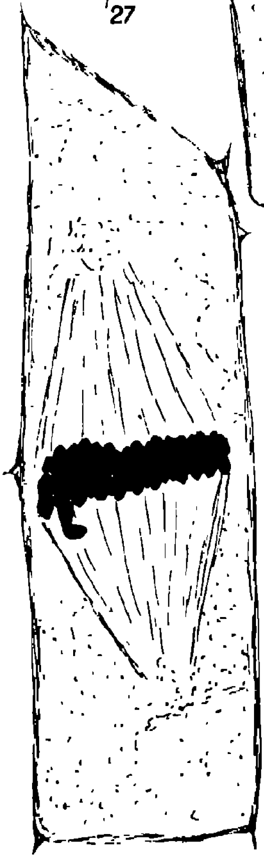

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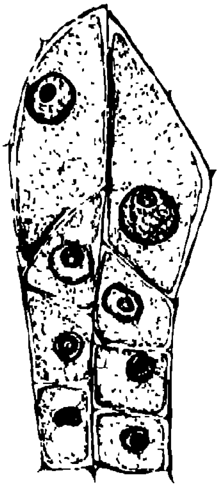

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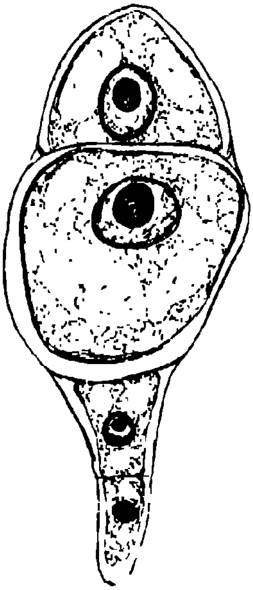

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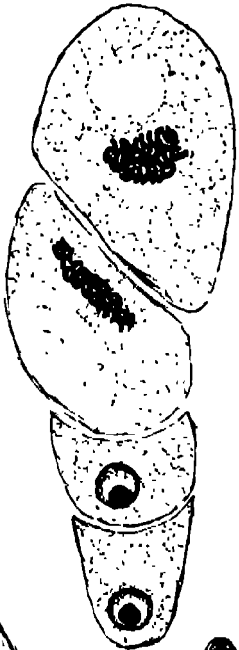

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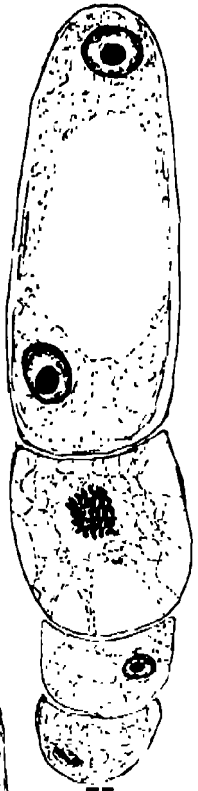

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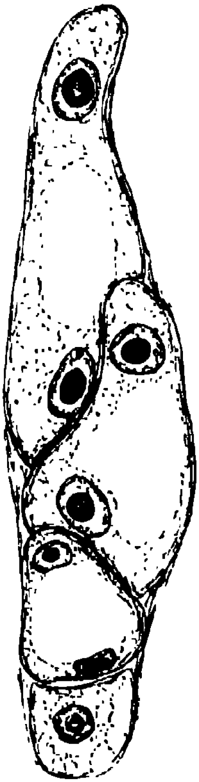

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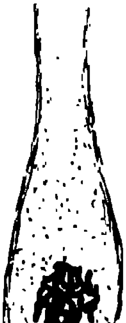

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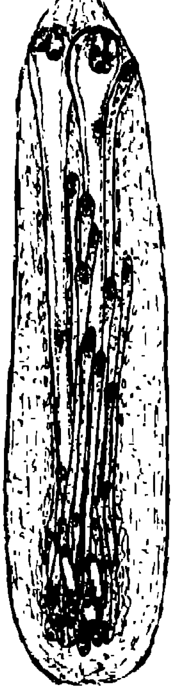

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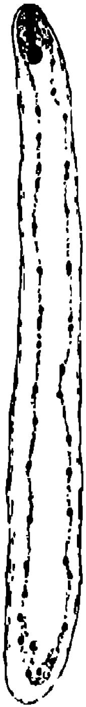

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PLATE 13.

FIG. 19.—Fertilised egg-cell in prophase for its first division, in the Tetraploid species *R. pendulina*, L., one synergid degenerating and the remains of the pollen tube above, a lining of free endosperm nuclei already formed.

FIG. 20.—Second division in the young embryo in the embryo-sac on the left, with lining of endosperm. On the right, a second embryo-sac degenerating, the two polar nuclei already degenerate, in the Tetraploid species *R. pendulina*, L.

FIG. 21.—Young multicellular embryo in the Diploid *R. palustris*, Marsh, the endosperm commencing cell formation.

FIG. 22.—Later stage in which the endosperm is forming cells in the upper and central parts of the embryo-sac, in the Tetraploid *R. Kotschyana*, Hort. Grav. (non Boiss.), an undescribed Linnean species of the Septet species BBDD.

FIG. 23.—Endosperm cells of the preceding more highly magnified.

FIG. 24.—Older embryo in the Hexaploid species *R. Sayi*, Schwein.

FIG. 25.—Divisions in the Enneaploid endosperm of the Hexaploid species *R. Sayi*, Schwein., showing 63 chromosomes as the result of the triple fusion of the first polar nucleus (21), the second polar nucleus (21), and the male nucleus (21).

Embryo-sac Formation in the Irregular Polyploid Species of Rosa, L.

PLATE 14.

FIG. 26.—Regular but unequal reduction in an embryo-sac mother cell of the irregular Tetraploid species *R. glauca*, Pourret, var. *dispersa* (Rouy) (*R. rubrifolia*, Vill., var. *dispersa*, Rouy): first division metaphase with 7 bivalent chromosomes on the equatorial plate and 14 univalents passing undivided to the micropylar pole.

FIG. 27.—Later stage in the irregular Tetraploid species *R. pomifera*, Herrm., var. *arvernensis* (Rouy) (*R. villosa* L. var. *arvernensis* Rouy): interkinesis with 7 chromosomes in the chalazal nucleus and 21 chromosomes in the micropylar nucleus.

FIG. 28.—First division metaphase in the embryo-sac mother cell of the Pentaploid species *R. glaucophylla*, Winch, var. *platysepala* (Rouy) (*R. glauca*, Vill., var. *platysepala* Rouy), with 7 bivalent chromosomes reducing on the equatorial plate and 21 univalents going to the micropylar pole.

FIG. 29.—Later stage in the Pentaploid species *R. uriensis*, Lag. et Pug., var. *pubescens*, R. Kell., in which the 7 bivalent chromosomes have reduced and one set of seven has gone to the chalazal pole, while the other set of seven has joined the 21 univalents at the micropylar pole, making an unequal reduction of chromosomes of 28 at the micropylar pole and 7 at the chalazal pole.

FIG. 30.—Second division metaphase in the Pentaploid *R. stylosa*, Desv., var. *Desvauxiana*, Ser. in D.C., with 7 chromosomes in the chalazal cell and 28 in the micropylar cell.

FIG. 31.—Equational and non-reductional division in an embryo-sac mother cell of the Pentaploid species *R. Froebelii*, Christ, var. *laxa* (Hort.), which might give rise to an apomictical embryo-sac (35 chromosomes).

PLATE 15.

FIG. 32.—Two megaspore quartets in the irregular Tetraploid species *R. mollis*, Sm., var. *typica*, W. Dod, showing the smaller chalazal cells and nuclei and the larger micropylar cells and nuclei, the uppermost enlarging to form embryo-sacs, pushing away the lower ones which are beginning to degenerate.

FIG. 33.—Megaspore quartet in the irregular Tetraploid species *R. pomifera*, Herrm., var. *vosgesiaca* (Rouy) (*R. villosa*, var. *vosgesiaca*, Rouy), in which the lower micropylar cell is enlarging apparently to form an embryo-sac. Note distinction in size between the two upper and lower cells and nuclei.

FIG. 34.—Megaspore quartet in the Pentaploid species *R. glaucophylla*, Winch, var. *brevipes* (Rouy) (*R. glauca*, var. *brevipes*, Rouy), showing metaphase plates in both of the upper and larger micropylar cells, each with 28 chromosomes, the reduced number.

FIG. 35.—Similar case in the Pentaploid species *R. mollissima*, Willd., var. *semitalis* (Rouy) (*R. tomentosa*, var. *semitalis*, Rouy), in which the upper micropylar cell is already 2-nucleate, while the lower one has a metaphase plate with 28 chromosomes, the reduced number.

FIG. 36.—Megaspore quartet in another variety of the Pentaploid species *R. mollissima*, Willd., var. *insidiosa* (Rouy) (*R. tomentosa*, var. *insidiosa*, Rouy), in which the upper and lower micropylar cells and the upper chalazal cell have carried through one division.

FIG. 37.—Metaphase plate of second embryo-sac division in the Pentaploid species *R. mollissima*, Willd., var. *insidiosa* (Rouy), with 28 chromosomes.

FIG. 38.—Embryo-sacs in the Pentaploid *R. agrestis*, Savi, var. *sepium* (Thuill.) (*R. sepium*, Thuill.): two fully grown 8-nucleate embryo-sacs are present, both showing adhesion of polar nuclei; there are also present one small 8-nucleate and several 4-nucleate and 2-nucleate embryo-sacs.

FIG. 39.—Embryo in the Pentaploid species *R. canina*, L., var. *nemophila* (Desegl. et Ozan.) (*R. nemophila*, Desegl. et Ozan.), with the normal lining of endosperm.

576 . 354 . 5 : 588 . 86 Oenothera

Meiosis in Oenothera purpurata *and* Oe. blandina.

By Professor R. Ruggles Gates, F.R.S., and K. M. Goodwin, B.Sc.

(Received May 27, 1931.)

[Plates 16–18.]

This paper is a study of the essential stages of pollen meiosis in two Oenotheras which show seven pairs of chromosomes in diakinesis and the heterotypic metaphase. Its intention is to throw further light on the origin of the pairs and their relation to the method of synapsis. Although these forms agree in having seven pairs of chromosomes and no linkage ring, yet they show certain characteristic cytological differences, which will appear in the description. The problem of the origin of chromosome linkage or catenation (Gates, 1931), so characteristic of Oenothera, is also involved.

Material and Methods.

Oe. purpurata was described by Klebahn (1925) from cultures derived from naturalised colonies of Oenothera on the Lunenburger Heide in North-western Germany. It is very much like *Oe. Hookeri*, but has small flowers. In crosses with *Oe. Hookeri* the smaller flowers of *Oe. purpurata* are stated by Rudloff (1929, *a*) to represent a monohybrid difference, the small flowers being dominant. By crossing it with various other forms, Rudloff found it to be homozygous, which he confirmed by showing that it had good pollen and seven free chromosome pairs in diakinesis.

Our seeds were obtained from Professor Klebahn in 1928, and grown in the Royal Botanic Gardens, Regent's Park. Owing to their habit of flowering very late, they failed entirely to progress beyond the rosette stage. Twenty-three plants were left in the ground over winter and when they formed shoots in the following June these were all strongly fasciated. The plants were devoid of red pigment until shortly before the flowering period. A red flush then developed at the base of the stem and passed upwards, pigment appearing meantime in the flower-buds. The fasciation was probably a result of the long period during which the rosettes had been carrying on photosynthesis and accumulating storage products. The later internodes, bearing flowers, and all the side branches as well were terete. The pollen was practically 100 per cent. good.

Oe. blandina was originally described under the name *Oe. Lamarckiana* mut. *velutina* (de Vries, 1917). It is like the *velutina* types found in twin hybrids, but with their characters more strongly expressed, and, unlike the *velutina* twin hybrids, it has no bad seeds. In a later paper, de Vries (1923) concluded that *blandina* had been derived from a half-mutant, *problandina*, which differs from it only in having about 25 per cent. of bad seeds, and which regularly produces about one-quarter *blandina*. The first specimens of *blandina* were recognised in 1904 in three plants derived in the third generation from *Oe. Lamarckiana lata* × *semilata*. *Oe. problandina* is regarded as derived from a *velutina* gamete of *Lamarckiana* chiefly by loss of its lethal factor, and it produces 25 per cent. of *blandina*. The latter is very uniform, except that in de Vries' cultures it has given rise to 0·1 per cent. of a mutant called *spiralis*. From seeds of de Vries, 33 plants of *blandina* were grown to maturity in 1930. The leaves are narrow and trough-shaped, like those of *velutina* hybrids, the stems are very hairy and the pigmentation redder than that of *rubrinervis*, the stems being very brittle. Examination of the pollen showed about 7 per cent. bad.

Young buds were fixed in Allen's Bouin at 38° C. after dipping in Carnoy. In the case of *Oe. blandina*, Allen's Bouin without chromic acid was also used, in order to alleviate the possible effects of chromic acid during the hour or more before the fixative could be changed. The sections were stained chiefly in Heidenhain's hæmatoxylin. In the case of *Oe. purpurata* various experiments were also made with smear preparations of the pollen mother cells. It is already known that Belling's method of smear preparations is not satisfactory for Oenothera. This is because of the thick cell walls, the presence of dark-staining substances in the cytoplasm, and of sticky contents in the anther. From this sticky fluid is afterwards developed the system of adhesive threads between the pollen grains which is so characteristic of Oenothera pollen. It was found that if the thick walls of the pollen mother cells are ruptured slightly, by squeezing out the contents of the anther with a scalpel, the dark-staining material can be washed out, leaving the cytoplasm clear and alveolar in appearance. Capinpin's (1930) method of squeezing the anthers between two slides was also used. The clarity of the smears was much improved by the use of picric acid as a clearing agent. Very good results were obtained by this method, as shown in figs. 11 to 17 and 20 and 21 (Plates 16 and 17), but the method is difficult to carry out and something is left to chance in rupturing the cell walls sufficiently to allow the staining substance in the cytoplasm to be removed, but not so much that the contents of the cell are lost.

Observations.

One or two longitudinal rows of polygonal pollen mother cells are present in *Oe. purpurata*, while a single row is commoner in *Oe. blandina*. In the resting stage of the nucleus it appears to be filled with a fine reticulum faintly stained, in the meshes of which are more densely staining bodies (figs. 1 to 4, Plate 16). As Miss Leliveld (1931) has recently described 14 such bodies in Oenothera, which fuse in pairs to form seven before synizesis, this matter was again examined with particular care. These masses occur chiefly near the periphery of the nuclear cavity, but they show no constancy either in number or size. Fig. 1, from *blandina* and fig. 2, from *purpurata*, are typical nuclei at this stage. Here the number of bodies should be 14. By way of comparison we include (fig. 3) a nucleus at the same stage from the haploid *Oe. rubricalyx* (see Gates and Goodwin, 1930). The number of bodies should be seven if they represent prochromosomes or chromocentres, but it is actually just as high as in the diploid nuclei. Fig. 4 is from another pollen mother cell nucleus of the same plant, showing a considerably lower number of these bodies. As they diminish in size through a graded series down to tiny granules which are often paler staining, it is usually impossible to determine a fixed number of such bodies in any particular nucleus. We therefore find no evidence that the number or size of these bodies is significant nor that they pair at any time.

Fig. 5 (Plate 16) is from a slightly later stage in the prophase of *blandina*, in which the reticulum threads have become coarser, the larger masses have disappeared and they appear to be scattered along the threads mostly in a beaded arrangement. During this period there is a general massing of the chromatin towards the periphery of the nucleus. As will be seen from these figures, the nucleolus is large and more or less centrally placed, usually vacuolated and often containing crystalloidal inclusions. Additional smaller nucleoli are occasionally present.

At a later stage the nucleolus takes up the well-known crescent shape and is attached to the nuclear membrane. The chromatin thread is much thicker and appears to be in direct connection with the nucleolus, as already described (Sheffield, 1927). These stages are undoubtedly extremely difficult to analyse in nuclei which are so small and in which the threads are so fine. The telosynaptic account of these stages has hitherto been partly an interpretation based upon the evidence derived from later stages. In a smear preparation obtained last summer, which has unfortunately since been lost, we obtained the first indications of parasynaptic pairing. The evidence has been considerably strengthened by the results presented in the present paper and the following one by Catcheside. Such evidence is very difficult to get, but its cumulative effect has been to lead us to adopt tentatively the parasynaptic hypothesis for Oenothera, thus bringing it into line with other forms. While there are still difficulties which can only be solved by further investigations, yet we at any rate have a number of definite observations which make it difficult to adhere to the telosynaptic theory.

These evidences of parasynaptic pairing have only been obtained under peculiarly favourable conditions. Generally our preparations fixed in Allen's Bouin have been in agreement with the full description for several species of Oenothera given by Sheffield (1927), but in the exceptional cases the additional evidence appears to require a change in the whole interpretation. Thus in one of the anthers of *Oe. purpurata* fixed in absolute acetic (fig. 6, Plate 16) the tapetal cells, although binucleate, as is normally the case at this time, differed from the normal in that the cells were not glandular in appearance but were relatively empty of cytoplasm. The cytoplasm of the pollen mother cells was also less granular than usual. As a result of this condition, the penetration of the fixing fluid to the pollen mother cell nuclei was undoubtedly more rapid, thus producing more perfect fixation of the threads. A portion of one of these nuclei is shown on a larger scale in fig. 7 (Plate 16). This represents the zygotene stage. In this preparation, all stages from early prophase to heterotypic metaphase could be observed. In some nuclei, as shown in fig. 7, the threads appeared to be in the process of pairing, certain threads being partly paired and partly divaricating, others paired along the traceable part of their length and still others in the same nucleus consisting of a single row of chromomeres. The single threads are, of course, conspicuously thinner than the double ones.

Another feature of this preparation is that the threads are loosely spread throughout the nuclear cavity and not drawn together into the condition known as synizesis. This confirms the view that synizesis is a condition of collapse and clumping of the delicate nuclear threads when the penetration is not sufficiently rapid and the fixation therefore imperfect.

At a later stage in the same preparation the threads have become shorter and much thicker; exhibiting irregular margins which are alternately light and dark, while the centre stains more lightly and looks slightly alveolar. It has hitherto been assumed that the threads at the stages represented by figs. 7

and 8 (Plates 16 and 17) were arranged end-to-end to form a continuous spireme. This is a point which is extremely difficult to determine by direct observation, because of the way in which the loops are aggregated. But from a critical study of stages a little later, in which ring pairs of chromosomes are linked into each other, it is necessary to conclude that the spireme is not really continuous at any stage, but that where chromosome pairs are free in diakinesis they are formed from the pairing and condensation of threads which were from the beginning separate from threads belonging to other pairs. This will be discussed later.

As the thickening threads continue to shorten they become more uniform in staining capacity and look flatter, more strap-like, but exhibit the dark outer edges and pale uniform centre. Following this stage the heavy thread becomes apparently looped (fig. 8), the number of "loops" corresponding with the haploid number of chromosomes. It is very difficult to determine with certainty from the stage represented by fig. 8 whether this is a continuous pachynema thrown into seven loops or whether it consists of seven circles (each representing a pair of chromosomes) interlocked with each other. From a critical study of various preparations at about this time, it is necessary to conclude that the latter interpretation is correct.

Incidentally, this cell and the adjacent ones in the row showed the probably abnormal condition of cytomyxis, a portion of the chromatin being extruded through an opening in the cell wallinto the next mother cell, where it forms a small rounded globule. In such cases the extrusion from several consecutive cells in the row takes place in the same direction, towards the top of the anther. Although the threads in fig. 8 are quite regularly arranged they are not of uniform width. Portions of two loops or rings (I and II) are very thick and show constrictions, but the thread shows signs of doubleness practically throughout and in some loops (III and VI) the arms are composed of two threads clearly twisted around each other.

The thickened double threads of fig. 8 appear to flatten as they shorten and widen still further to form the chromosomes. This stage is represented by figs. 9 and 10 (Plate 17), in which there are fine and sometimes long connections between flattened chromosomes with pointed ends, which still show indications of their double origin in that their margins are darker and the centre lighter, as though they were formed by the lateral fusion of two threads. There is never, however, any appearance of the threads being twisted about each other at this stage. The connections all appear single, but they are so fine and so near the limits of resolution that a visible doubleness should perhaps not be expected. Figs. 6 to 10 and 19 (Plates 16 and 17) are all from the same flower bud, which showed the peculiar tapetum. Other buds from the same plant, as well as all the material collected from other plants in this culture, showed the usual condition of seven chromosome pairs in diakinesis and heterotypic metaphase. But the bud in question differed also in showing chromosome linkage. In fig. 9 the condition could be most fully analysed. The nucleus shows clearly a ring of five and two independent chains of three chromosomes. In addition there is one pair and one single chromosome. This is the first time an odd number of linked chromosomes has been found in a plant with 14 chromosomes. Another peculiarity of this nucleus is that the middle chromosome (*abc*) in the upper chain of the three is connected at one end by fine threads to both the other chromosomes. How such a condition has arisen or what exactly is its meaning is not clear, but the result is that chromosomes *b* and *c* both show clearly two connections at one end. Chromosomes I, 3, *a* and *c*, show definite evidence that they are composed of two parallel halves. Fig. 10 shows similarly a chain of five or perhaps six chromosomes, but the nucleus was cut. Other nuclei (cut) were found with chains of three chromosomes, but never in this slide the normal condition of seven pairs. Fig. 19, from the same slide, is a heterotypic metaphase with a chain of five and two chains of three, showing that it must have been derived from a diakinesis stage like that in fig. 9. The chromosome arrangement 5 + 3 + 3 is therefore probably characteristic of this bud. The number of chromosomes linked end-to-end in this bud appears to be variable, but that chains of seven chromosomes occur is certain. Free pairs of chromosomes also occur, which are not ring but chain pairs.

There is no possibility that the bud showing these peculiarities was derived by accident from some other Oenothera culture, for *Oe. purpurata*, being grown as a biennial, came into flower early in 1930, and when these buds were collected there were no other Oenotheras which had reached the stage when collections could be made.

The diakinesis stages in figs. 11 to 17 (Plates 16 and 17) are from smear preparations of *purpurata* anthers. In some of these the nuclear membrane was invisible (and the cell wall only is represented). Seven ring pairs are usually present, but there may be at least one rod pair, as in figs. 13 and 15, or certain ones may be clearly arranged side by side, as in figs. 15 and 16. Occasionally a ring pair may be drawn out into a thin ring, as in fig. 11. This is probably done in making the smear. The pairs are frequently all free from each other (none interlocked), as in fig. 17, but often one or more of the ring pairs are linked into each other. In no case have all the ring pairs in *purpurata* been found interlocked. This condition is in contrast with that in *blandina* (*vide infra*). Four ring pairs is the highest number we have found interlocked in *purpurata* (fig. 14).

In the heterotypic metaphase these chromosome pairs are arranged on the spindle in various ways corresponding to the arrangements during diakinesis, except that in metaphase ring pairs are rarely found to be interlocked. This is apparently because when pairs are interlocked in diakinesis the rings usually open before metaphase, allowing the pairs to separate. A ring pair which has opened in this way probably always remains open and forms either an open ring or a rod pair. In fig. 18 (Plate 17) five of the metaphase pairs are rings and two are rods. Fig. 19 represents the unusual condition in which thread connections which are presumably persistent from diakinesis remained between chromosomes of different pairs. Figs. 20 and 21 (Plate 17) are from the same stage in smear preparations. The spindle fibres are invisible, and the chromosome connections are seldom seen.

A brief comparative description may now be given of conditions in *blandina.* Fig. 22 (Plate 17) represents a post-zygotene stage where some threads are much thicker than others, the chromatin representing one or more ring pairs being in a more advanced stage of development than the rest of the spireme.

Fig. 23 (Plate 17) represents the typical " second contraction " stage of the pachynema, which has hitherto been regarded as a continuous spireme thrown into loops and containing the 14 chromosomes arranged end-to-end, except in cases where one or more free pairs were precociously segmented off. In this figure, the number of " loops " is probably seven. One of them, which is darker, represents a pair of more condensed chromosomes in top focus, and apparently free from the rest. It is believed that the remainder of the " spireme " represents six pairs of interlocked chromosomes in an uncondensed condition, and not twelve chromosomes arranged tandem.

Figs. 24 to 32 (Plate 18) show various arrangements of the chromosome pairs in diakinesis. It has hitherto been supposed that the pairs were formed by segmentation of the pachynema represented in fig. 23, but critical examination fails to reveal stages of segmentation between pachynema and diakinesis. On the other hand, comparison of such figures as 23 and 32 leads to the conclusion that in the former the " spireme " is really composed of interlocked ring pairs, which gradually condense into the condition of more or less interlocked pairs in diakinesis. A point of this kind can only be determined with certainty in forms having seven free pairs, and in such forms it appears that there is no continuous spireme at any stage. This is one of the most cogent pieces of evidence leading to a change from the telosynaptic to the parasynaptic point of view.

It will also be seen from figs. 24 to 32 that the amount of interlocking of the ring pairs with each other varies greatly. All the pairs may be free and independent, as in figs. 26, 28 and 29, or at the other extreme all may be interlocked, as in fig. 24. What were formerly regarded as pairs precociously cut off from the spireme are now recognised as pairs which have been free throughout the prophase stages because the two members have paired with each other without becoming entangled with other pairs. Interlinkage of ring pairs is then to be regarded as a condition which arises when members of different pairs become linked with each other in the process of parallel pairing of chromosomes in the long thin delicate thread stage of early heterotypic prophase.

The arrangement of pairs in diakinesis in both *blandina* and *purpurata* has been studied statistically, and the results are presented in Table I. It will be seen that there are characteristic differences between the two forms. In *blandina* 26 per cent. of the pollen mother cells in diakinesis have seven free rings, while *purpurata* has 57·4 per cent. The corresponding values at heterotypic metaphase are 53·6 per cent. and 88·3 per cent. respectively. This shows that some of the interlocked ring pairs must open out and become free from each other between diakinesis and metaphase. The rest of the table shows that practically all the interlocked arrangements are more frequent in *blandina* than in *purpurata*, and this is true both of the diakinesis and metaphase stages. Thus, while both forms have seven ring pairs, these are more frequently interlocked in *blandina* and more generally free from each other in *purpurata*.

Fig. 33 (Plate 18) represents the multipolar spindle stage in *blandina*. In this cell four ring pairs are interlocked, two are free and one a rod pair.

In the heterotypic metaphase in *blandina* there is again considerable variation, as shown by figs. 34 to 36 (Plate 18), but pairs are rarely found interlocked at that stage. In fig. 34 (Plate 18) all the pairs are rings except one which is a rod. Fig. 35 (Plate 18) is of special interest because all the pairs are rods, and one of them shows double connections, with a small granule upon each which suggests an earlier chiasma. In fig. 36 (Plate 18) there is again a single rod pair and some of the spindle fibre attachments are clearly visible.

Discussion.

A number of points arise from the facts here presented, and some of them may be briefly discussed.

(1) In addition to the end-to-end arrangement of several chromosomes to form a chain or a closed ring, which has long been known in Oenothera and has recently been called catenation (Gates, 1931), it is now necessary to recognise the frequent occurrence of a double chain of interlocked pairs—a construction precisely like that of an iron chain composed of links (see *e.g.*, figs. 24, 25). This has been observed before, but never in such frequency as found here. As already suggested, this interlinkage evidently arises in connection with the process of lateral pairing of the chromosome threads. We may suppose the chromatin threads to be in a state of more or less active oscillatory movement throughout the stages of mitosis and meiosis, and that this, together with the pairing beginning at the ends of the chromosomes and proceeding towards their middle, will lead to some pairs being interlocked.

The evidence indicates that in *blandina* one ring pair is frequently separate while the others still form an apparently continuous spireme, or, as we now consider, interlocked pairs. This shows that the process is not entirely a haphazard one. In *Oe. ammophila*, which normally has a ring of twelve chromosomes and one free pair, this pair is also frequently independent at an equally early stage (Sheffield, 1927), while the remainder form a continuous spireme which will condense and segment into a ring of 12 chromosomes. In *blandina*, on the other hand, the other 12 chromosomes are ring pairs frequently interlinked. It is further to be noted that while interlinkage of ring pairs is a common occurrence, yet it is extremely rare for two ring pairs to be interlinked with the same (third) ring pair. Davis (1909), in a paper on a form of *Oe. grandiflora* with seven ring pairs, also found them sometimes free and sometimes interlinked.

Returning to the question of ring pairs, which is the normal form of the chromosomes during diakinesis both in *blandina* and *purpurata*, according to the theory of terminalisation of chiasmata (Darlington, 1929) these will only be formed when there is at least one chiasma on each side of the point of spindle fibre attachment of the chromosomes composing a pair. From all that is now known of the split in the chromosomes in ordinary mitosis, we may conclude that these heterotypic prophase chromosomes are already split at the time of pairing. Since in both *blandina* and *purpurata* the normal condition is seven ring pairs in diakinesis, it is necessary to conclude that chiasmata to the number of at least two in each pair of chromosomes are of general occurrence in Oenothera. They may, of course, be of much greater frequency, but the only direct evidence of this is in fig. 8, where the threads composing each loop appear to be closely twisted around each other.

By reference to Table I it will be seen that among 191 nuclei of *blandina* in diakinesis, a single rod pair of chromosomes occurs in each of eight nuclei. As already mentioned, the metaphase rod pairs are probably for the most part rings which have opened out in separating from other rings. But when occurring in diakinesis, a rod pair presumably means that there was no *chiasma* between the point of spindle fibre attachment and one end of the parasynaptic thread pair. The failure to form a chiasma will then have occurred once in each of eight nuclei among a total of 191. Since each nucleus has 14 chromosomes, and no nucleus has been found with more than one rod pair in diakinesis, the frequency of such failure of chiasmata would be

$$8 \div (191 \times 14) \times 100$$

$=$ 0·3 per cent., or three times in 1000 chromosomes. The indirect as well as the observational evidence thus indicates that the chromosomes are closely twisted about each other, so that there will nearly always be at least one chiasma between the point of spindle fibre attachment and each end of the chromosome.

Perhaps the most important theoretical result of the view that the chromosomes are closely twisted about each other in heterotypic prophase and that chiasmata are of frequent occurrence, is its effect on the theory of chromosome individuality during meiosis. It was, until recently, held generally that chromosome individuality was maintained during meiosis as during somatic divisions. But if at least two, and probably several, chiasmata are formed in almost every pair of chromosomes, and these are accompanied by crossing-over, then chromosome individuality may be said to have largely broken down at this stage.

The difference between *blandina* and *purpurata* as regards the interlocking of the seven ring pairs has already been pointed out. Interlocking at diakinesis is nearly twice as frequent in *blandina* as in *purpurata*, while the frequency of interlocked pairs is higher in *blandina* at the heterotypic metaphase than in *purpurata* at diakinesis, notwithstanding that a number of interlocked rings have opened and separated between diakinesis and metaphase. The higher frequency of interlocking in *blandina* may be supposed to result from a greater mingling of the chromosomes during interkinesis, as a result of which the

Table I.—Frequency of Chromosome Arrangements.

Arrangement of chromosomes.	Diakinesis.				Metaphase of first division.			
	Oe. blandina.		*Oe. purpurata.*		*Oe. blandina.*		*Oe. purpurata.*	
	Per cent.	No. of cells.	Per cent.	No. of cells.	Per cent.	No. of cells.	Per cent.	No. of cells.
7 free ring pairs	26·0	50	57·4	66	53·6	67	88·3	83
7 linked ring pairs	3·1	6	—	—	—	—	—	—
6 linked ring pairs, 1 free ring pair	11·0	21	—	—	—	—	—	—
5 linked ring pairs, 2 linked ring pairs	4·1	8	—	—	—	—	—	—
5 linked ring pairs, 2 free ring pairs	12·5	24	5·2	6	—	—	—	—
4 linked ring pairs, 3 linked ring pairs	3·1	6	—	—	—	—	—	—
4 linked ring pairs, 3 free ring pairs	10·4	20	4·3	5	—	—	—	—
4 linked ring pairs, 2 linked ring pairs, 1 free ring pair	4·5	9	5·2	6	—	—	—	—
3 linked ring pairs, 4 free ring pairs	5·8	11	5·2	6	—	—	—	—
3 linked ring pairs, 2 linked ring pairs, 2 free ring pairs	3·1	6	—	—	—	—	—	—
2 linked ring pairs, 5 free ring pairs	9·4	13	0·8	1	—	—	—	—
2 linked ring pairs, 4 free ring pairs, 1 rod pair	0·5	1	—	—	—	—	—	—
2 linked ring pairs, 2 linked ring pairs, 3 free ring pairs	3·6	7	8·7	10	—	—	—	—
6 free ring pairs, 1 rod pair	3·6	7	2·6	3	23·2	29	9·5	9
5 free ring pairs, 2 rod pairs	—	—	—	—	19·2	24	2·1	2
4 free ring pairs, 3 rod pairs	—	—	—	—	3·2	4	—	—
3 free ring pairs, 4 rod pairs	—	—	—	—	0·8	1	—	—
Total number of cells examined	—	191	—	115	—	125	—	94

members of the pairs are more widely or more often separated from each other than in *purpurata*. It would then be expected that as pairing begins to take place in the early heterotypic prophase there will be a greater chance of two pairs becoming entangled with each other in the process of synapsis or pairing. There is still some difference of opinion as to the frequency or generality with which the chromosomes maintain a paired arrangement in the metaphase of somatic mitosis in plants. Probably the pairing is closer in some plants and looser in others, occurring in some plants with no greater frequency than would be expected according to the laws of chance.

Such a range of conditions appears clearly to exist in different animals. If a similar difference in the strength of attraction between members of each pair occurs in the case of *blandina* and *purpurata*, then we might expect to find the chromosomes more noticeably paired in the metaphase plates of *purpurata* than in *blandina*. Examination of somatic mitoses in these two forms, however, fails to furnish any evidence that visible pairing is a more common occurrence in *purpurata* than in *blandina*. Similarly, in a previous study (Gates and Sheffield, 1929) of catenation in *Oe*. (*biennis* × *rubricalyx*) × *ammophila* and its reciprocal *Oe*. *ammophila* × (*biennis* × *rubricalyx*), the former showed seven pairs of chromosomes in meiosis while the latter had three pairs and a ring of eight. But no evidence was obtained of any corresponding amount of pairing of chromosomes in the somatic divisions of the two forms.

If the somatic pairing of chromosomes is closer in some organisms than in others—and there seems no escape from this conclusion—then we must assume that some selective mutual attraction, probably determined by the individual chromomeres, leads to the paired condition in somatic prophases and metaphase, and that such an attraction is stronger in some organisms and tissues than in others. If that is the case, then there is no reason for relying solely upon chiasmata as the cause of homologous chromosomes remaining paired in the heterotypic prophases.

Finally, with regard to parasynapsis and telosynapsis, Oenothera has been almost universally regarded as telosynaptic for many years, and numerous authors, including all the most careful workers, have adhered in all essentials to the telosynaptic account originally published (Gates, 1908). Observations suggesting the possibility of parasynapsis have only recently begun to appear. Catcheside (1931) has recently brought evidence from a triploid Oenothera which is incompatible with a telosynaptic view, but even in the present paper it is only under exceptionally favourable conditions that direct evidence in favour of parasynapsis has been obtained, both in the early zygotene stage and in the stage of "second contraction" just before diakinesis. Even here, conclusive evidence could only be expected in forms like the present ones with seven chromosome pairs and no large rings. In *Oenothera rubrinervis*, in which the telosynaptic account was first clearly given, there is now known to be constantly a ring of six chromosomes end-to-end, and it was natural that these should be regarded as six of the somatic chromosomes which entered upon meiosis. The parasynaptic view makes it necessary to suppose that these really represent chromosome threads which have paired laterally only at their ends, so forming a ring of six chromosomes which may then be supposed to correspond as individuals with the six somatic chromosomes which entered upon meiosis, except that crossing-over has taken place in their relatively homozygous terminal portions. This matter is more fully discussed by Catcheside in the paper which follows. Kihara (1927), from a study of synapsis in Rumex and *Oenothera biennis*, put forward the somewhat similar hypothesis that after parasynaptic pairing the chromosomes open out in such a way as to form ring complexes of different types.

If all the phenomena of ring formation in Oenothera species and hybrids can be made to fall in with the conception of parasynapsis, then the hypothesis will undoubtedly be strengthened, but it is not necessary to discuss this aspect of the subject further here. Other recent papers, such as that of Fikry (1930) on Rumex, and that of Maeda (1930) on Lathyrus, tend to strengthen the view that parasynapsis is of general occurrence in plants. The latter paper, together with that of Latter (1926) on Lathyrus, also lead to the conclusion that according to the method of fixation one may obtain an essentially telosynaptic or parasynaptic story from preparations, the fusion of delicate parallel threads in certain fixatives giving an appearance of telosynapsis. If that is the case in Lathyrus, it may be used as an argument in favour of parasynapsis in Oenothera, where the threads in zygotene stage are much more delicate and easily fused together to give a misleading appearance of telosynapsis, especially in the numerous forms with a large ring of chromosomes which have, until now, been mainly studied.

Summary.

The critical stages of meiosis have been investigated in *Oenothera blandina* and *Oe. purpurata*, both of which have seven free pairs of chromosomes in meiosis. There is a characteristic difference in the amount of interlocking of pairs during diakinesis in the two forms. In 26 per cent. of the pollen mother cells of *blandina* all the pairs were free from each other, while 57 per cent. of the pollen mother cells of *purpurata* were in this condition. This difference is interpreted as due to the fact that in the early heterotypic prophases of *blandina* the chromosomes of a pair are generally less near to each other and the threads are hence more liable to become entangled with those of another pair in the process of pairing. This difference would ultimately be due to a stronger attraction between homologous chromosomes at meiosis in *purpurata* than in *blandina.*

Similarly in heterotypic metaphase, 54 per cent. of mother cells in *blandina* showed no interlocking of pairs, while in *purpurata* 88 per cent. were free from interlocking. This and other evidence shows that in both forms some of the interlinked rings separate by the opening out of ring pairs between diakinesis and heterotypic metaphase.

Definite evidence of parasynapsis in Oenothera has been found. The best evidence concerning the zygotene stage is obtained from a bud of *purpurata* in which the tapetal cells were non-glandular, allowing rapid penetration. In the pollen mother cells of this bud a ring of five chromosomes as well as other catenations were present. Pairing of divaricated threads was seen in the zygotene stage, and some evidences of doubleness in the stages following. In the "second contraction" stage of the pachynema the spireme is not continuous as hitherto supposed, but seven loops representing the pairs of chromosomes are more or less completely interlocked, thus giving the appearance of a continuous spireme, except when certain pairs are free from the beginning. Each loop condenses into a ring pair of chromosomes, which may remain interlocked with other similar loops. Between diakinesis and metaphase, some of the ring pairs open and thus become separated. The continuous pachynema hitherto observed in Oenothera has been due to the presence of a number of linked chromosomes in a ring or chain, while free pairs of chromosomes, when present, are frequently linked round the chain but never actually continuous with it.

Evidence is obtained that the double threads making up the loops of the pachynema stage are closely twisted about each other, allowing for the occurrence of numerous chiasmata. Rod pairs of chromosomes, which occur in *blandina* with a frequency of about 3 in 1000, indicate that occasionally there is no chiasma between the point of spindle fibre attachment and an end of a chromosome. Thus we may conclude that while the ends of chromosomes in Oenothera pair side by side, asynapsis characterises their median portions.

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EXPLANATION OF PLATES.

All figures were sketched at table level with the aid of a camera lucida. A 2 mm. Zeiss apochromatic objective (N.A. 1·4) was used. Zeiss oc. × 20 (magnification × 2950) was used for figs. 1 to 5, 7 to 10, 18, 19, 22 to 36; Zeiss oc. × 15 (magnification × 2000) employed for figs. 11 to 17, 20, 21 (smear preparations); Zeiss oc. × 10 (magnification 1350) for fig. 6.

Figs. 11 to 17, 20 and 21 represent the whole contents of pollen mother cells, but in figs. 12, 15 and 16 the nuclear membrane has not been shown (smear preparations).

Figs. 6 to 10 and 19 are from a preparation of *Oe. purpurata* fixed in absolute-acetic, the rest in Allen's Bouin (Kihara's method).

The drawings have been reproduced without reduction. They are all from pollen mother cells.

Plate 16.

Fig. 1.—The resting nucleus in *Oe. blandina*, showing densely staining masses in the reticulum.

Fig. 2.—The resting nucleus in *Oe. purpurata*, showing densely staining masses of various sizes.

Fig. 3.—The resting nucleus in haploid *Oe. rubricalyx* showing about 16 darkly staining masses in the reticulum.

Fig. 4.—A resting nucleus from the same anther as fig. 3, showing only 6 darkly staining masses in the reticulum.

Fig. 5.—Very early prophase in *Oe. blandina* showing coarser reticulum and scattered beaded threads, especially towards periphery of nucleus.

Fig. 6.—Zygotene in *Oe. purpurata* (absolute-acetic preparation). Section of whole anther showing non-glandular binucleate tapetum, 3 pollen mother-cells with diffuse threads.

FIG. 7.—Zygotene. A portion of top right-hand nucleus in fig. 6 showing progressive pairing of threads.

FIGS. 13 to 16.—Diakinesis in smear preparations of *Oe. purpurata*.

FIG. 13.—5 free pairs, 1 rod pair, 2 interlocked pairs of chromosomes.

FIG. 14.—4 interlocked pairs, 2 interlocked pairs, 1 free pair.

FIG. 15.—2 rod pairs, 2 interlocked pairs, 3 free pairs, 3 nucleoli.

FIG. 16.—5 free pairs, 2 interlocked pairs; 1 free pair shows evidence of earlier chiasma.

PLATE 17.

Oe. purpurata.

FIG. 8.—Later stage in *Oe. purpurata* (absolute-acetic) showing thickened thread, 7 loops of double thread of uneven thickness, showing twisting. Note cytomyxis.

FIGS. 9 and 10.—Post-pachytene stage in *Oe. purpurata* (absolute-acetic) showing flattened chromosomes with indications of their double origin and very fine connections.

FIG. 9.—Shows a ring of 5 chromosomes (1–5), 2 independent chains of 3 chromosomes, I–III and *a*, *b*, *c*, where chromosome *b* is connected to both *a* and *c*; 1 free pair and 1 single chromosome. Fig. 10 shows a cut nucleus with a chain of 5 chromosomes.

FIG. 11.—Diakinesis in *Oe. purpurata* (smear), 7 pairs of chromosomes, 4 interlocked pairs (1 of them drawn out), 2 interlocked pairs and 1 free pair.

FIG. 12.—Diakinesis in *Oe. purpurata* (smear), 2 of the pairs interlocked.

FIG. 17.—Diakinesis in *Oe. purpurata* (smear), 7 free pairs.

FIG. 18.—Heterotypic metaphase in *Oe. purpurata*, 5 ring pairs (1 broken) and 2 rod pairs of chromosomes.

FIG. 19.—Heterotypic metaphase in *Oe. purpurata* (absolute-acetic) showing a chain of 5, a chain of 4, a chain of 3 and 1 free pair of chromosomes.

FIG. 20 and 21.—Heterotypic metaphase in smear preparations of *Oe. purpurata*.

Oe. blandina.

FIG. 22.—Post-zygotene in *Oe. blandina* showing progressive thickening of loops.

FIG. 23.—"Second contraction" in *Oe. blandina*, showing 1 free pair of condensed chromosomes in top focus and interlocked loops.

PLATE 18.

Oe. blandina.

FIG. 24.—Diakinesis. 7 free pairs of chromosomes interlocked.

FIG. 25.—Diakinesis. 6 pairs of chromosomes interlocked and 1 free pair.

FIG. 26.—Diakinesis. 7 free pairs of chromosomes.

FIG. 27.—Diakinesis. 5 free pairs, 2 interlocked pairs of chromosomes.

FIGS. 28 and 29.—Diakinesis. 7 free pairs (1 ring open in fig. 29).

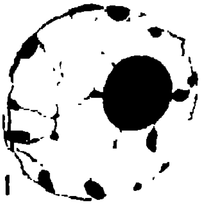
FIGS. 30 to 32.—Slightly later than fig. 23. Fig. 30 shows 4 interlocked pairs and 3 interlocked pairs (one out). Figs. 31 and 32 show 1 free pair, the rest interlocked.

FIG. 33.—Multipolar spindle. 4 interlocked pairs of chromosomes in a chain, 2 free ring pairs and 1 rod pair.

FIG. 34.—Heterotypic metaphase. 7 free pairs of chromosomes (1 a rod pair).

FIG. 35.—Heterotypic metaphase. 7 free rod pairs of chromosomes. 1 has a double connection and 2 granules, suggesting earlier chiasma.

FIG. 36.—Heterotypic metaphase. 7 free pairs of chromosomes; 1 rod chiasma and 6 rings.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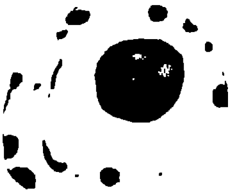
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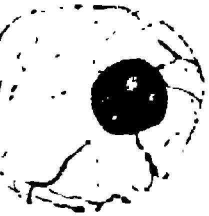
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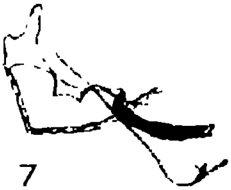
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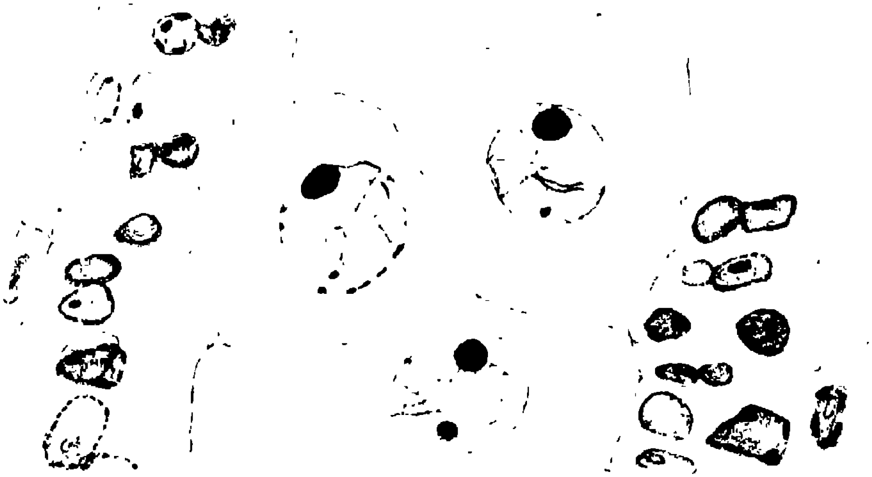
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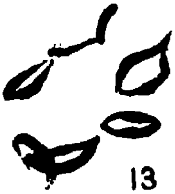
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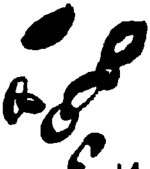
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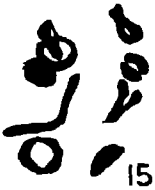
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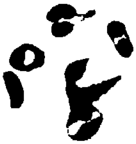
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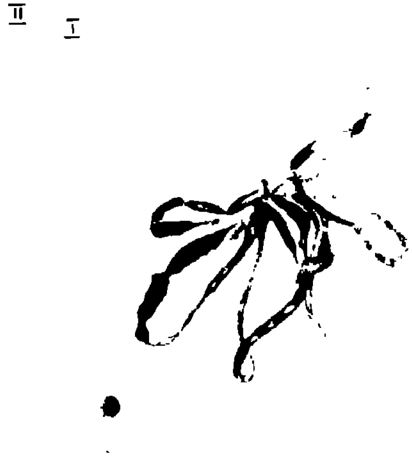

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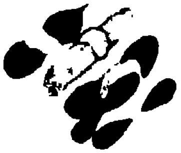

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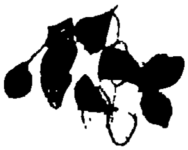

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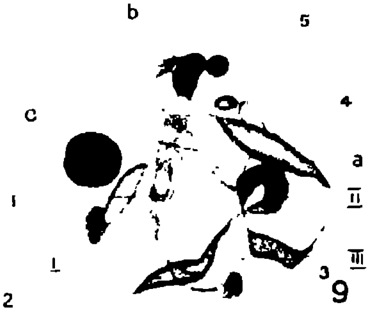

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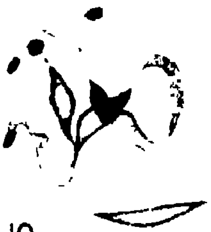

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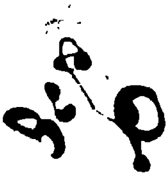

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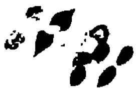

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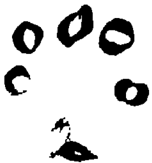

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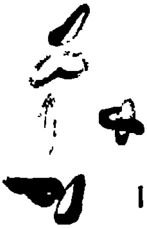

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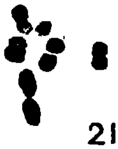

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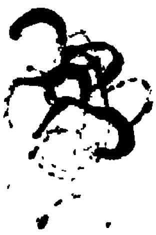

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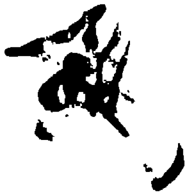

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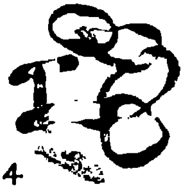
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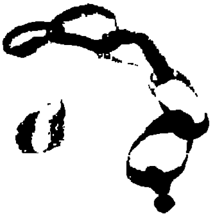
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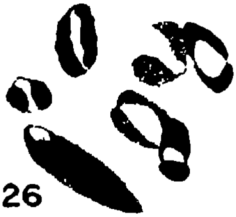
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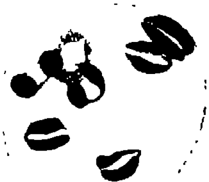
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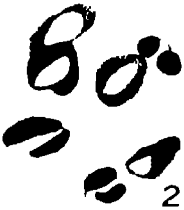
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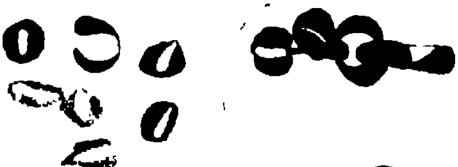
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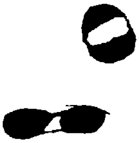
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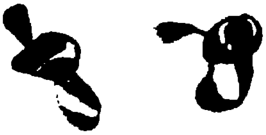
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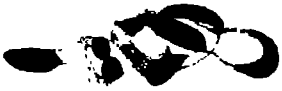
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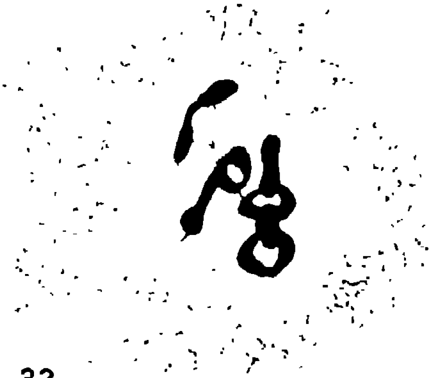
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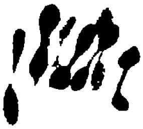
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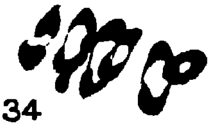
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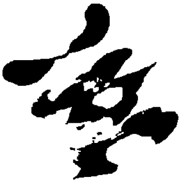
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576 . 354 . 46 : 588 . 86 Oenothera

Critical Evidence of Parasynapsis in Oenothera.

By D. G. Catcheside, M.Sc.

(Communicated by R. Ruggles Gates, F.R.S.—Received May 27, 1931).

Introduction.

The formation, in *Oenothera* species, of rings or chains of chromosomes joined end to end, first found by Gates (1908), was shown by Cleland (1922) to be characteristic for particular forms. In *Oe. franciscana*, for instance, there is at diakinesis and metaphase regularly a ring of 4 chromosomes and 5 bivalents, and in *Oe. muricata* (Cleland, 1926), there is a ring or chain of 14 chromosomes. Numerous workers have added to the number of species, mutants and hybrids examined, and Gerhard (1929) has presented a classified list. It is apparent, then, that every *Oenothera* form has a specific chromosomal configuration or, more precisely, that each form has a particular configuration which is more frequent than any other and from which other possible configurations may be derived by simple modifications.

Undoubtedly, therefore, there must be some innate reason for the fixity of construction, and Darlington (1929) traces it to interchanges of end-segments between non-homologous chromosomes occurring during the evolution of the various forms. Each species is thus a structural hybrid, true breeding by reason of the operation of a balanced lethal system. Pairing is essentially parasynaptic and takes place between homologous end-segments with subsequent terminalisation of chiasmata established between the paired segments. In this way, chromosome segments must be specific in their position and attraction, a matter that has been adequately proved by Blakeslee and Cleland (1930) and Cleland and Oehlkers (1930).

Parasynapsis, however, has not been properly demonstrated in *Oenothera*, and no description so far given fits the facts of specificity of attraction. Boedijn's (1925) description in *Oe. Lamarckiana* of the irregular formation, near the end of diakinesis, of chains from seven pairs previously formed, is contrary to the established fact that this species has regularly a ring of 12 chromosomes and a bivalent (Cleland, 1925 and 1929; Håkansson, 1926). Kihara (1927) based a parasynaptic account on the doppelketten origin of a ring of six in *Rumex acetosella*, but the case is not strictly comparable since that plant is a polyploid. Kâto (1930) derives the ring of 12 chromosomes in *Rhoeo discolor* from a similar double chain, but such an explanation in *Oenothera* will not fit in with the genetical facts or with specificity.

Triploid *Oe. pycnocarpa* has supplied evidence (Catcheside, 1931) that diploid *Oe. pycnocarpa*, having a ring of 14 chromosomes, is a structural hybrid; moreover, the occurrence of triple (compound) chiasmata, at the point where three chromosomes pair together at one end, has proved that each chromosome is already equationally split.

The present work has been done upon a form that early promised to show evidence of homologous end segments and of interstitial chiasmata, and from it a suggested account of meiosis in *Oenothera* has been drawn up.

Material and Methods.

The material was derived from one plant of a culture grown by Professor R. R. Gates, F.R.S., from seed supplied by Professor Hugo de Vries, under the name of *Oe. Lamarckiana* mut. *pallescens*, which is a trisomic; it was soon seen, however, that this plant had 14 chromosomes, organised as a ring of 6 and 4 ring bivalents. The plant thus appears to be a diploid form derived from the trisomic and is perhaps a half-mutant; this can be decided solely from its breeding behaviour.* Buds of various ages, stripped of their sepals, were fixed in Allen's modification of Bouin, after about 3 to 5 seconds' treatment with Carnoy, following Kihara's method; though immersion in Carnoy removes the wax and thus materially aids wetting of the material by the aqueous fixative, yet the fluid tends to mar the staining properties of the cells, so that its use is not to be recommended unequivocally. The material was cut at 15 microns, after embedding in 49° wax, and stained with Heidenhain's iron-alum hæmatoxylin.

Observations.

Numerous nuclei at diakinesis and the heterotypic metaphase show a regular organisation of the chromosome complement into a circle of six and four bivalents; occasionally, as in many other *Oenothera* forms, an open chain replaces the ring due, as it is now believed, to failure of chiasma formation between the chromosomes involved. The expected doubleness of the connection and of the chromosomes, due to the longitudinal split into two chromatids (Catcheside, 1931), has been seen to some extent; careful focussing at both diakinesis and metaphase reveals double ends to the chromosomes, while in some cases two fine strands connect adjacent chromosomes in the ring (fig. 3, A). The bivalents, moreover, reveal the same tetrad structure that has been observed constantly in many other forms. Naturally, not all the chromosome ends will appear double, and some will be clearer than others, owing to the fact that there will be all gradations from the case in which the two chromatids are lying side by side in the plane of the field to that in which they are superposed; in the latter case, the distance separating them being already very small, to all appearance they will coincide. Failure to observe the double connection may be traced to two causes: (1) the extreme delicacy of the threads, owing to which collapsing effects of fixatives are liable to be frequent, and (2) the very small distance separating the two threads, so that, except with critical illumination and the most accurate microscopy, diffraction effects will blur their distinctness and make them appear single. In fact, in a connection between two chromosomes, the distance between the two threads is small compared with the wave-length of light; it will therefore be usually impossible to separate the two threads completely throughout their length. It is evident, however, that there is more than sufficient proof of their existence, both from the split chromosome ends in this diploid and more clearly in the triple connections shown in trisomics (Håkansson, 1930) and triploids (Catcheside, 1931). Fig. 3, A, shows a very clear double connection in the chain.

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* The F_2 progeny, this summer, shows a splitting into 2 types, one repeating the half-mutant, the other a new form.

At the earliest phase as yet successfully analysed, the four bivalents, in favourable nuclei, may be found as separate entities organically connected neither to each other nor to the ring of six chromosomes. The stage shown at fig. 1 expresses in all essentials the principles of chiasma formation at diplotene required on the theory of parasynapsis and of structural hybridity. The movement of the chiasmata towards the ends of the chromosomes probably has already commenced. It will be advantageous to describe the sequence of changes in the bivalents and in the circle of six separately, and to deal with the bivalents first, since much is known of bivalent construction in other forms.

The most frequent number of chiasmata seen in the bivalents at diplotene is three (fig. 1, *b*, *c* and *e*), though two (fig. 1, *d*) or four and as many as five (fig. 2, *a*) are occasionally present. In the cases seen, the chromosomes have the major portion of their substance located between two chiasmata, suggesting that (1) chiasma formation is most frequent towards the ends of the bivalents, and that (2) there is a movement of chiasmata away from this portion (containing the attachment constriction) towards the much more slender distal portions. The individual chromatids may be seen along practically the whole length of the bivalent, except, that is, in regions where they are superposed in the axis of the microscope; it is evident, too, that sister chromatids are paired together at the attachment constriction. At this time they are very variable in thickness and outline, being thick at their centres and thinner distally; the surface in the thick portion is more or less irregular, while in the thinnest distal portions an appearance suggestive of a row of variously sized chromomeres may be distinguished in some of the less condensed bivalents. The scarcity of stages (such as those shown at fig. 2, *b* and *c*) between this one and diakinesis, when the chromosomes are fully condensed, suggests that the processes of terminalisation of chiasmata and of condensation and smoothing of chromosomes proceed relatively rapidly. Stages indicate, however, a reduction in number of the chiasmata to the two normal at diakinesis. In the greater proportion of cases the two chiasmata are both terminal; quite frequently, however, one (fig. 2, *d*, *f*, *g*) and rarely both (fig. 2, *e*) are subterminal, there being a smaller or larger portion of the bivalent distal to the chiasma,

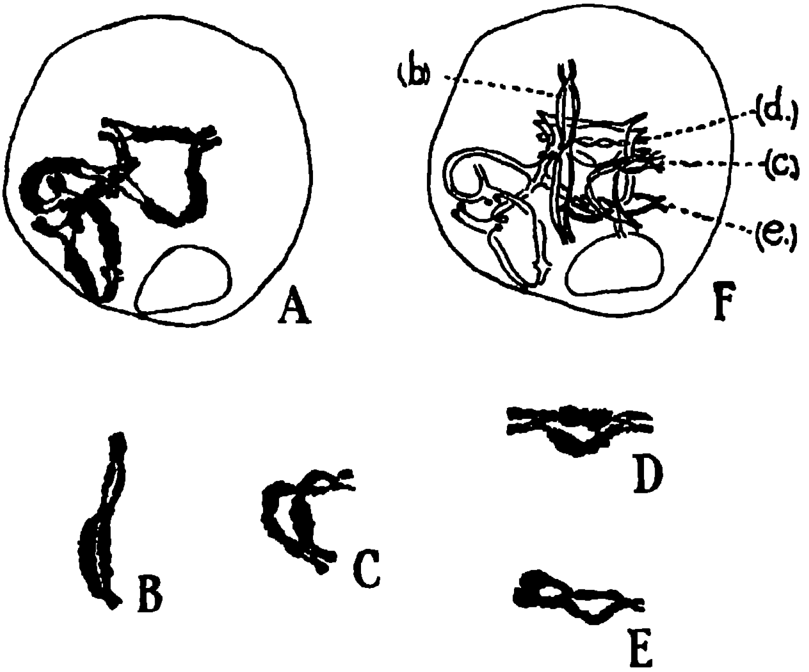

Fig. 1.—Diplotene, probably rather late, the constituent parts drawn separately, their relative positions being indicated at (*F*); (A), ring of six, showing interstitial chiasmata with varying lengths of chromatid distal to the chiasma; (B)–(E), the four bivalents, showing interstitial chiasmata. × 3300.

due, I believe, to incomplete terminalisation, rather than to arrest by a change in homology. This opinion is based upon the greater frequency of such interstitial chiasmata at diakinesis than at the heterotypic metaphase. Interstitial chiasmata are seen at this latter stage (fig. 2, *h*) and later such bivalents lag behind others in which the chiasmata were both terminalised; such lagging is evidently due to the unravelling of the chiasma, movement of the chiasma being caused by the disjunction of pairs of chromatids joined together at the attachment constriction.

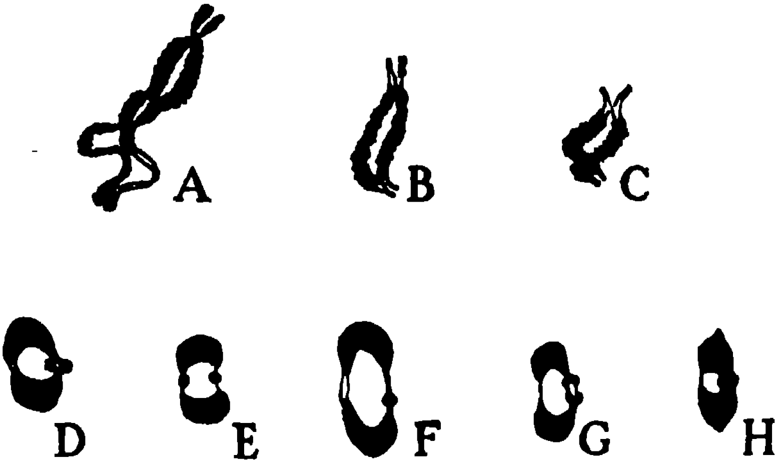

FIG. 2.—Bivalents at various stages: (*a*), diplotene with 5 chiasmata; (*b*) and (*c*), early diakinesis, tetrad structure evident; (*d*–*g*), diakinesis showing presence of interstitial chiasmata; (*h*), heterotypic metaphase, with interstitial chiasma. Figs. A, D, E, F, H, × 3300; figs. B, C, G, × 2700.

The behaviour of the ring-forming chromosomes is similar in principle, except that the chiasmata do not occur along so great a length of a particular chromosome. So far as one can decide, a single chiasma links adjacent chromosomes, and occurs fairly near to the ends of the chromosomes associated by it (fig. 1, A). The length of chromatids distal to the chiasma appears to differ slightly around the ring, suggesting that the lengths of the homologous end-segments differ in different cases, or that the chiasma is itself formed at random in the length of the end segment; more probably it is a combination of both. The present material is insufficient to assess the relative importance of these two factors. If the chiasmata are established at varying distances from the ends of the segments and assuming that the rate of movement of the chiasmata towards the terminal position is the same in all cases, it is readily understood why in some instances at diakinesis the chiasma is fully terminalised while *in others it* is more or less interstitial, showing a larger or smaller distal segment. Such structures have been seen frequently, in the present material, in the ring of chromosomes (fig. 3, A, B, F); but at the heterotypic metaphase,

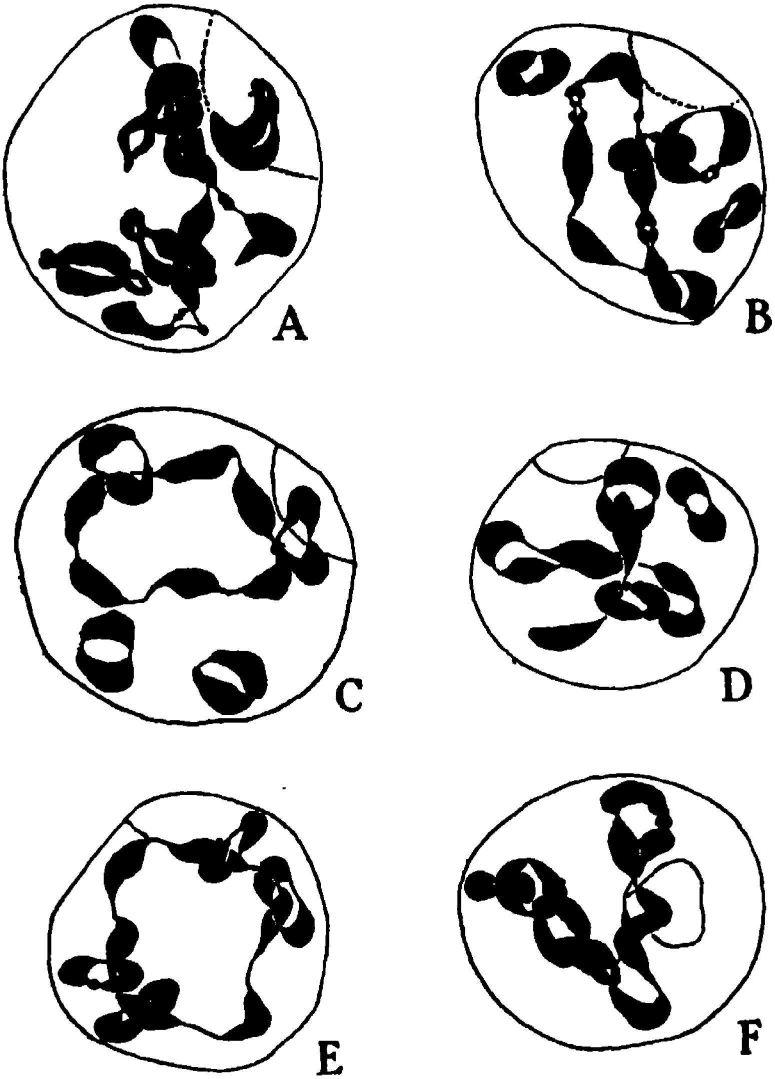

FIG. 3.—Whole nuclei at diakinesis: (*a*), early diakinesis, showing double connection in chain and interstitial chiasmata in chain and bivalents; (*b*), interstitial chiasmata and showing double connections; (*c*), bivalent clipped on to ring between two chiasmata terminalised in same direction; (*d*), chain of six rod bivalent and three ring bivalents; (*e*), four bivalents interlocked independently with the ring; (*f*), ring and four bivalents interlocked like the links of a chain. Figs. A–C, × 3300; figs. D–F, × 2700.

owing to continued terminal movement of the chiasmata during the whole of diakinesis, practically all of the chiasmata are quite terminal; rarely a slightly interstitial one is seen (fig. 4, C).

Bearing upon the orderly and uniform rate of movement of the chiasmata in a terminal direction is the observation that at very early diakinesis, while markedly interstitial chiasmata are usual in the bivalents, such are proportionately less frequent in the ring. This accords with the observation that the chiasmata are formed at random along a considerably smaller extent of the more distal regions of the ring-forming chromosomes, the latter thus having a considerably shorter distance to travel. Active movement of the chiasmata in this manner must mean a distinct liveliness on the part of the nuclear material, and may explain why in many plants this stage is so sensitive to the effects of certain fixatives.

A remarkable feature is the frequent interlocking of the ring bivalents with the circle of six or with each other. This is especially noticeable at diakinesis, though it can be traced through the multipolar spindle stage to the heterotypic metaphase. Although interlocking makes for a close group at metaphase, often difficult to analyse completely, it does not seem to interfere with the regular arrangement (in respect of disjunction) of the ring and bivalents on the spindle. Accommodation of the interlocked bivalents is sometimes

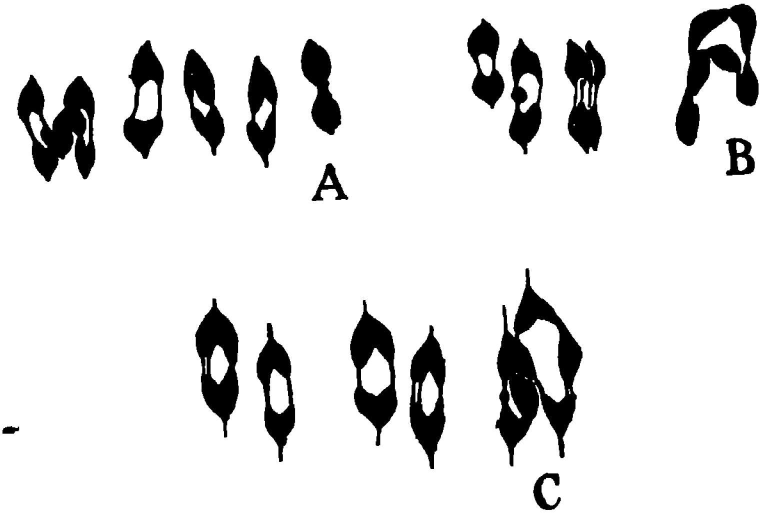

Fig. 4.—Heterotypic metaphase: (*a*), regular disjunction in the ring; (*b*), double non-disjunction on same side in ring, two bivalents interlocked and one bivalent with interstitial chiasma; (*c*), non-disjunction in ring, interstitial chiasma in ring, and double connections shown in some cases. Figs. A and B, × 2700; fig. C, × 3300.

possible only by their arrangement at different levels on the spindle. A statistical study of the various types of interlocking has been made at diakinesis; unfortunately, the difficulties of observation at metaphase have prevented any extended study of the question at that stage. Incidentally, the frequency of failure of chiasmata at diakinesis had also been estimated.

Illustrations of various types of interlocking are seen at diakinesis at fig. 3, B, C, E and F. Fig. 3, *e*, shows all four bivalents interlocked independently with the ring; while fig. 3, F, shows the four bivalents and the ring interlocked like the links of a chain. In fig. 3, C, one bivalent (to the right) is clipped on to the ring between two chiasmata terminalised in the same direction. Interlocking is difficult to trace at metaphase, but in fig. 4, B, two of the bivalents are thus entangled. Failure of chiasma formation between two of the chromosomes in the ring is seen at fig. 3, A, while there is in addition failure to form more than a single chiasma between the two chromosomes of a bivalent at fig. 3, D.

In 331 whole nuclei examined the following classes of arrangement were found :—

		No. of nuclei.	Per centage.
Ring of 6 and 4 ring bivalents, 14 diakinesis chiasmata....		292	88·2
Chain of 6 and 4 ring bivalents	13 diakinesis chiasmata	19	11·2
Ring of 6, 3 ring bivalents and 1 rod bivalent		18	
Chain of 6, 3 ring bivalents and 1 rod bivalent	12 diakinesis chiasmata	1	0·6
Chain of 4, chain of 2 and 4 ring bivalents		1	
Total		331	

In all, 21 different classes of interlocking were found among the 292 nuclei with a ring of 6 and 4 ring bivalents; the other 39 nuclei could be grouped into 13 classes.

It is evident then that the chance of failure of one chiasma per nucleus is $11\cdot2 + (0\cdot6 \times 2) = 12\cdot4$ per cent. Hence the chance of failure of two chiasmata together in one nucleus is $(12\cdot4)^2/100 = 1\cdot54$ per cent.; the 0·6 per cent. actually found is a rather poor agreement with the expectation arrived at thus, and is probably due to the small number of nuclei counted. The comparative chances of failure of any particular chiasma in the ring and the bivalents, considered separately, are respectively $22 \times 100/331 \times 6 = 1\cdot1$ per cent., and $19 \times 100/331 \times 8 = 0\cdot7$ per cent., indicating that chiasmata are formed over a greater length in the bivalents as compared with the ring-forming chromosomes.

A detailed enumeration of the different classes of interlocking is quite unnecessary; the results are summarised in Table I, an interlock counting once only and that in conjunction with the particular structure concerned in the interlock.

Now, the maximum possible interlocks with the ring is 292 × 4, and also the maximum possible interlocks of bivalents with each other is 292 × 3. Hence the chances of a bivalent interlocking with the ring or with another bivalent are respectively:—

$$\frac{465}{4 \times 292} \times 100 \qquad \text{and} \qquad \frac{111}{3 \times 292} \times 100$$

i.e.,

$$39 \cdot 5 \text{ per cent.} \qquad \text{and} \qquad 12 \cdot 6 \text{ per cent.}$$

Hence the relative chances of a bivalent interlocking with the ring or with another bivalent are as 3·13 : 1, suggesting that the relative chance is conditioned by the relative lengths of (or numbers of chromosomes, viz., 3 : 1, taking part in) the ring of 6 and a bivalent.

The total average amount of interlocking found in this *Oenothera* is about

Table I.—A summary of interlocking in 292 nuclei, in which a particular interlock is counted once only in connection with the structure (ring or bivalent) with which it is made.

Interlocks of bivalents with the ring of six.			Interlocks of bivalents *inter se*.	
No. of interlocks.	**No. of nuclei.**	**Total interlocks.**	**No. of interlocks.**	**Total interlocks.**
0	18	0	0	0
	15	0	1	15
	4	0	2	8
	1	0	3	3
1	62	62	0	0
	37	37	1	37
	5	5	2	10
	1	1	3	3
2	65	130	0	0
	21	42	1	21
	5	10	2	10
3	46	138	0	0
	4	12	1	4
4	7	28	0	0
Totals	—	465	—	111

50 per cent., a circumstance which suggests that interlocking is quite at random. If the chromosomes are distributed at random throughout the resting nucleus, any particular chromosome will have an equal chance of approaching its mate from a position either between or without any other pair of mating chromosomes. The chance of a particular interlock occurring would therefore be 50 per cent.; this should be the proportion of interlocks established no matter what the chromosomal configuration may be.

Discussion.

The recent work of Håkansson (1930) on trisomics, of Capinpin (1930), and of Catcheside (1931) on triploids has added materially to the body of facts that fit in with the interchange theory. The segmental interchange theory now offers, then, a unified explanation of (1) ring formation, (2) its inheritance on selfing and crossing, (3) the origin, by occasional crossing over, of mutants differing in properties of ring formation from their parents, (4) the chromosomal configurations of triploids and of trisomics, and (5) the specificity of attraction of chromosome segments as shown by hybridisation experiments.

An exact demonstration of parasynapsis, however, is not to be found in the literature, though certain structures figured by Håkansson (1930), under the title "Querarm," are obviously the result of the formation of interstitial chiasmata and thus provide good evidence that side by side pairing of the chromosomes forming the bivalents had taken place. In the published figures, too, the fine connections have always been represented as single strands and the two chromatids constituting a single chromosome never shown, as though the equational division of the chromosomes was delayed until after the anaphase of the first division. In the early interkinesis nucleus the two chromatids of a chromosome separate from one another at their ends and pivot at their attachment constriction to form a characteristic Maltese cross; the equational division is, however, unlikely to occur so late and especially in the *condensed* chromosomes. It must be concluded, therefore, that the division of the chromosome into two chromatids must have occurred at some period during the prophase of meiosis; this division has never been traced in spite of a great deal of work upon this stage, nor have workers displayed much curiosity concerning its history. The present study shows that it must precede diplotene, and possibly it takes place during the period represented in preparations by the synizetic knot, which is undoubtedly an artefact produced by the collapsing effects of the fixatives at a time when the chromosomes are in a delicate thread-like condition. That the connections between any two chromosomes, and hence the chromosomes themselves, must be double follows from the presence of two connections proceeding from each chromosome when three of them are associated by their ends; this has been demonstrated clearly in trisomics and triploids. The lobed ends of the chromosomes often show clearly, and in favourable cases the doubleness of the connections between chromosomes is traceable in the *Oenothera* here reported.

On the basis of parasynapsis and segmental interchange, fairly definite situations may be predicted for various prophase stages of meiosis in *Oenothera*. The six ring-forming chromosomes typical of the present form are shown diagrammatically at leptotene in fig. 5, *a*; at this stage they would be free from

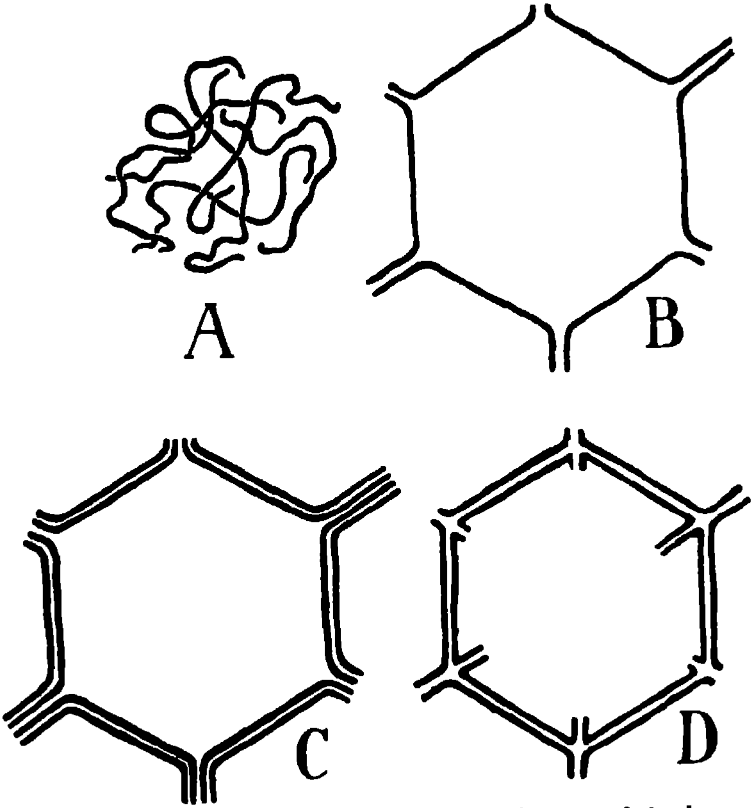

FIG. 5.—Diagram of prophase of meiosis in *Oenothera* with a ring of six chromosomes: (*a*) leptotene, the thread-like chromosomes unpaired and separate; (*b*) zygotene, parasynaptic pairing having taken place between homologous end segments of chromosomes, their lengths differing around the ring thus established; (*c*) pachytene, the chromosomes are split into two chromatids; crossing-over occurs at this stage; (*d*) diplotene, chiasmata having been formed at one point in each of the paired segments, with a frequency decreasing from the ends of chromosomes; note the difference in lengths of the distal regions.

one another as slender threads lying in the nucleus. It must be noted, in the first place, that each of the chromosomes may be divided into three parts, a central segment that may be homologous with portions of the central segments of one or more other chromosomes, and two end segments that have different specific attractions, one of them for one end of another chromosome, the other for one end of a third chromosome. For the present, it is impossible to assume that the central segment has no homologous portions in other chromosomes; hence, judging from the later stages (diakinesis) that are known sufficiently well, it is necessary to asume that pairing at zygotene (fig. 5, *b*) is restricted almost entirely to the end segments of the chromosomes; so far as the published data go, it might be said that restriction is complete. In the case of the ring-forming chromosomes, it might well be that synapsis of the chromosomes normally begins at their ends, as in *Mecostethus* (Janssens, 1924) and other Orthoptera, thus mechanically restricting the pairing to the end segments, since the proximal ends of the segments are marked by a change to different homologies; the interlocking of bivalents with each other or with the ring, seen later on during prophase, would be susceptible of easy interpretation on such a basis. It should be noted that the lengths of the homologous end segments vary. After synapsis, presumably, each chromosome divides longitudinally to form the two chromatids characteristic of pachytene (fig. 5, *c*); subsequently, chiasmata would be developed interstitially, probably no more than two being developed normally in each chromosome, one in each of the two end segments (fig. 5, *d*). Movement of such chiasmata towards the ends would mean the steady diminution in size of the portions distal to the chiasma, until finally they had become terminal, as is generally the case at diakinesis and at the heterotypic metaphase in *Oenothera*.

Failure of chiasma formation at any one point must signify a break in the ring at that point. It has been deduced above, from the observations made, that failure of one chiasma occurs in an average of 12·4 per cent. of nuclei; hence the chance of failure of any particular chiasma, normally present at diakinesis, is 12·4/14 = 0·9 per cent.

Incomplete terminalisation must result in characteristic appearances, such as those described above, of interstitial chiasmata at the later stages of meiosis. Examination of the published figures shows a decided paucity of such structure while their true nature does not seem to have been recognised. Sheffield (1927), in *Oe. novae-scotiæ*, at diakinesis (fig. 23), describes a granule on the connection between two chromosomes; this is evidently a subterminal chiasma, with a very minute distal region. Cleland and Oehlkers (1930) show structures, at diakinesis, that appear to me suggestive of interstitial chiasmata, in *Oe. Cockerelli* (fig. 4, *b*) and in *Oe.* (*strigosa* × *Lamarckiana cruciata*) *deprimens gaudens* (fig. 12, *i*).

The presence of an interstitial chiasma always causes marked lagging at anaphase, since the completion of the terminal movement of the chiasma requires an appreciable amount of time. When the chiasma is fully terminalised, the breakage of the connection is a rapid process, and then the ends of the chromosomes, freed from the restraining influence of the connection, snap away from one another. Illustrations of these phases are well shown at figs. 51 and 32 in a paper by Gates and Sheffield (1929). Unfortunately, late anaphases and telophases are unrepresented in my own material.

Bivalents, in forms possessing them, provide a rather different case; observations have shown that the two chromosomes of a bivalent are usually associated at diakinesis and metaphase by terminal chiasmata. Interstitial chiasmata have been figured by Håkansson, and these suggest immediately that the typical terminal chiasmata result from terminalisation of originally interstitial ones. From genetical and other considerations, there is every probability that the two chromosomes constituting a bivalent are homologous throughout their length; cytologically, this is essential only in a perfectly stable form. We have then, in the bivalents, a probable history as follows: association side by side of two chromosomes throughout their length, synapsis commencing at the ends (zygotene); splitting of each to give four chromatids in all (pachytene); random formation of chiasmata along the length of the bivalent with subsequent movement of them away from the spindle attachment point to either end of the bivalent, until normally they are quite terminal. In some cases, *e.g.*, Håkansson's bivalents with crossarms, the terminalisation process is, for some reason or another, incomplete, whence the appearance of interstitial chiasmata at diakinesis or still later stages.

Various facts, however, lead one to believe, with Newton and Darlington (1930), that the formation of chiasmata is not, for some reason, random throughout the whole length of the bivalent, but that chiasmata occur with decreasing frequency as the attachment constriction is approached. In harmony with this view is the fact that bivalents with relatively subterminal chiasmata, with but a small distal arm, are much more frequent than those with decidedly interstitial chiasmata.

The terms used extensively in *Oenothera* literature for different prophase stages of meiosis, are not those used in the present paper, nor in many other accounts of the reduction division in different organisms. The following equivalents are suggested as being approximately correct :—

1. Presynizesis	Leptotene.
2. Synizesis	Zygotene.
3. Open spireme	Pachytene.
4. Pachytene and second contraction.	Diplotene and movement of chiasmata.

Two of these, namely, numbers 2 and 4, are stages at which the nuclear contents, in a relatively fine thread condition, must be in a very lively state of movement ; in the former stage the chromosomes are actively approximating and pairing, while in the latter there is looping and subsequent rippling of the chromatids as the chiasmata are terminalised. At these periods, then, the nuclear contents must be in a remarkably sensitive state, and hence the deleterious effects of fixatives just a trifle slow to act are not at all surprising. The very frequent interlocking of bivalents *inter se* and also with the ring of six chromosomes must also tend to foster the frequent appearance of a second contraction into a single more or less dense mass. That interlocking is, however, a mechanical accident occurring at the time of the pairing of homologues must be fairly evident.

The agreement of the cytological observations with the genetical conditions, as adduced by Renner and his co-workers, is sufficiently obvious ; but the likelihood of a genetical map being constructed to correspond to the cytological conditions seems, however, to be exceedingly remote. In *Oenothera* there must be a regular segregation of sister chromatids together, under the control of the spindle fibres, not only in the region of the spindle fibre attachment, but also in other regions, even though the latter are paired with other chromosomes which are influenced in their migration by synapsis in still other regions with other chromosomes in turn. The cytological evidence suggests that this is the case ; indeed, the regular zigzag arrangement of alternate chromosomes in the ring, with chiasmata usually terminal or almost so, permits of no other interpretation, but precise genetical evidence on this point is difficult to obtain. Muller, however, in the case of the " Star Curly " translocation in Drosophila, in which a large portion of chromosome III is attached to chromosome II, has shown (1930) that the directions of migration of the free fragment of chromosome III and of the homologous chromosome III of normal structure are correlated with or influenced by the position and direction of the chromosome II bearing the translocated part of chromosome III. The result was that 1·7 times as many gametes were formed bearing normally proportioned chromosome combinations as those bearing disproportioned combinations; assortment was thus determinate rather than random, a point in agreement with the metaphase arrangement of the ring-forming chromosomes in *Oenothera*.

The history of the chromosomes and their chromatids during the prophase of meiosis is now quite clear from detailed work on critical material (*Tulipa, Hyacinthus, Lilium*, etc.); at pachytene the bivalents consist of four chromatids, and at diplotene loops are developed by apparent separation of the chromatids in pairs alternately in adjacent internodes, the node being marked by a chiasma. Most are agreed that the point of exchange of chromatid segments is actually at the chiasma; moreover, detailed genetical evidence (largely from *Drosophila*) shows that cross-overs occur between only two chromatids at any one point, thus destroying Janssen's theory of total chiasmatypy.

Sax (1930) regards the chiasmata as primarily points of interchange of partners among the paired chromatids, supposing that close association and coiling of the paired chromatids prevents any movement of the chiasmata and further that any strain imposed on the chiasma is liable to cause the crossed chromatids to break at the point of contact. Strains are imposed, he states, by (1) further opening out of the internodes, (2) unequal contraction of the two chromonemata, or (3) a slight amount of twisting of the chromonema as it coils. The breaks occur at the same locus in most cases owing to the close association of the paired chromatids; subsequently, the free ends of the chromatids thus broken pair, gene by gene, with the intact chromatids until the broken ends of the different chromatids are brought into contact. Thus the number of chiasmata is reduced by crossing-over, the remaining ones persisting until they are pulled apart at anaphase. On the basis of this, Sax accounts for Sturtevant's (1925) case of unequal cross-over at the bar locus of the X-chromosome in *Drosophila*, and for the crossing-over characteristics of triploid *Drosophila* as compared with the normal diploids. This speculation, of course, fails to explain the formation of chiasmata. Moreover, it takes no account of the facts of the frequent terminal movement of chiasmata and of their rarer movement towards the attachment constriction in *Fritillaria* (Newton and Darlington, 1930); the evidence in this *Oenothera* points to regular terminalisation.

Darlington (1931), dealing with crossing-over and chiasma formation, has advanced an hypothesis which regards diplotene chiasmata as originating at points where an exchange has previously occurred between non-sister chromatids. Cross-overs between sister chromatids, while feasible, would not show themselves as chiasmata. It is evident, therefore, that the exchanges cannot occur later than pachytene. Bridges and Anderson's (1925) work on triploid *Drosophila* females shows clearly that crossing-over occurs when each of the three chromosomes is in the two-strand stage. Hence, the genetical evidence proves that it must occur at pachytene when each chromosome is split into two chromatids. Darlington (1930) has presented critical evidence, from tetraploid *Hyacinthus*, that a change in identity must occur at a chiasma. He showed that where two chromatids that formed a chiasma with two other chromatids, also formed chiasmata with a third pair of chromatids on either side of the first chiasma, the chromatids must then be assumed to be in the same combinations on the two sides of this chiasma ; this must involve crossing-over at the first chiasma. Such configurations were found in tetrasomic hyacinths and also tetraploid *Primula sinensis*, and provides a clear demonstration of chiasmatypy. The difference in cross-over value between male and female *Drosophila* is suggested by Darlington (1931) to be correlated with localisation of chiasmata in the heterogametic sex.

Various mechanisms have been suggested by which it has been supposed crossing-over might occur. In common with Morgan (1925), Belling (1928) has postulated that breaks in the chain of genes are most likely to occur at leptotene, with the homologous chromosomes already split, when it is drawn out into a highly tenuous thread. He assumed that breaks would occur at random and that the chance of coincidence of two breaks in two particular chromatids at any particular point is $1/x$; and showed that $1/x = C/100$, where C is the observed cross-over percentage between two particular adjacent genes. His hypothesis makes the assumptions that the breaks occur in the chain of genes ; that the breaks are at random in each of the two sister strands, except that there is a certain minimum and maximum limit between consecutive breaks ; and that when two breaks coincide the two broken left-hand ends reunite at random with the two broken right-hand ends, so that either the original ends reunite or a chiasma is formed. This theory fails to explain why the occurrence of one break should interfere with the occurrence of a second ; nor why crossing-over should vary with temperature in the manner demonstrated by Plough (1917). At present, then, there is no satisfactory explanation of crossing-over, though the fact of its occurrence is thoroughly established both genetically and cytologically.

Interlocking of bivalents and rings in *Oenothera* is perhaps due in the first instance to the association of homologues commencing at their two ends and proceeding towards their centres, interlocking then depending upon the chance disposition of the homologues at either side of another non-homologous chromosome, with the result that at pachytene the situation would be such as is shown diagrammatically in fig. 6, *a*. Chiasmata are perhaps formed at random in the bivalents, with a frequency decreasing towards the spindle fibre attachment constriction; moreover, sister chromatids remain in contact at this constriction. The simplest case is shown in fig. 6, *b*, in which a single chiasma is formed on either side of the attachment constriction and either side of the interlocked chromosome; this case is explicable either on the classical theory of separation of pairs of chromatids in reductional and equational planes in succeeding internodes, or on the chiasmatype theory. The remaining two cases (fig. 6, *c* and *d*) illustrated are explicable solely upon the latter theory. Alternate equational and reductional exchanges are impossible in these cases because, presumably, the interlocking chromosome provides a complete barrier to an exchange in this manner in certain regions. Therefore, as will readily be seen, the chiasmata at X in fig. 6, *c* and *d*, must represent an actual crossing-over between the associated pairs of chromatids; presumably, too, the remaining chiasmata likewise are the consequence of cross-overs. A configuration of the type in fig. 6, *c*, is shown in fig. 3, *c*; such cases are rare and have been seen only three times in 331 nuclei at diakinesis.

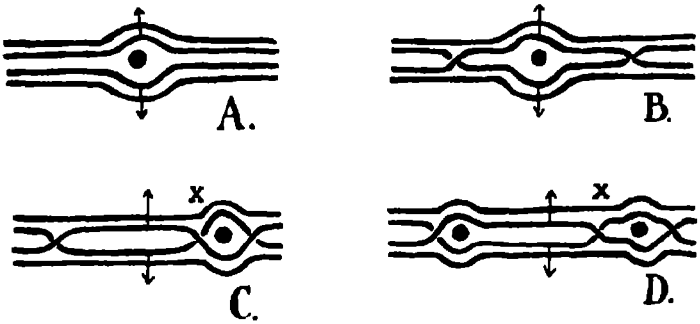

FIG. 6.—Occurrence of interlocking, the interlocked portion of the ring being represented by a black spot, and the attachment constriction by the arrows: (*a*) pachytene; (*b*)-(*d*) diplotene; (*b*) one chiasma either side of attachment constriction and of interlocked ring; (*c*) two chiasmata one side of attachment constriction and either side of interlocked ring; (*d*) same as last, but with a further interlock in the region of the attachment constriction. Figs. (C) and (D) prove that the chiasma at X is the result of a cross-over.

It is necessary, at this point, to consider in how far genetical results justify the suggestion that cytological chiasmata represent points at which genetical crossing-over has occurred between two chromatids. The crucial point here is, of course, to discover whether there is a good agreement between the number of chiasmata actually observed and those possible upon genetical data; for this purpose all that it is really necessary to know is the full length of the genetical chromosomes in any one form and also the cytological conditions. This cannot, at present, be realised fully,[*] but *Drosophila* offers the best opportunities. According to Morgan, Sturtevant and Bridges (1925) it has four genetical chromosomes (Nos. 1 to 4) respectively, 70, 107·5, 106·2 and 0·9 units (centimorgans) in length; a prediction of the possible numbers of chiasmata in each of the bivalents is thus possible. For the present, it seems preferable to neglect the fourth (small) chromosome and to consider only the other three. In these three, the attachment constriction is nearly terminal, near locus 70, in chromosome I (the X-chromosome) and approximately median, near loci 54·5 and 48 respectively, in chromosomes II and III. The map distance between any two points represents, of course, the expected cross-over value under more or less standard conditions of environment and genetical composition (freedom from modifiers, etc.); moreover, the cross-over value is the percentage of gametes that show that particular crossing-over. Bridges and Anderson (1925) found that a fairly frequent class of flies in the progeny of triploid females was one in which the two X's were duplicates of each other for part of their length, but not for the remaining part. This result indicated that each of the original chromosomes was in a two-strand stage when crossing-over occurred. Crossing-over thus takes place between only two of the four chromatids of a bivalent at any one point. Hence, a particular cross-over value between any two points represents an actual crossing-over (or chiasma) in twice as many gamete mother cells. For instance, crossing-over must occur in 100 per cent. of gamete mother cells, between two points 50 units of map distance apart. The apparent number of chiasmata is, however, reduced by double crossing-over. It is well known, also, that double crossing-over is reduced by interference, the amount of the effect being stated as the inverse numerical proportion and is derived from observed value of cross-over ÷ expected value of cross-over, which is called the observed coincidence of double crossing-over. According to Weinstein (1917) coincidence increases, beyond the initial region (nearly 15 units long) of zero value, until at a distance of 40 units it is slightly in excess of unity, beyond which it again decreased as far as it could be followed. The decrease in coincidence beyond a maximum, indicates that the distance between two successive cross-overs in the same pair of chromatids tends to be of a particular length, namely 40 *centimorgans*. Hence we must expect a cytological chiasma to occur in a pair of chromatids at points whose distance apart corresponds to 40 units of the genetic chromosome. Therefore, in *Drosophila* it is to be expected that chromosome pair I will have an average of slightly less than two chiasmata to the left of the attachment constriction and practically none to the right of it; with terminalisation of the chiasmata the two X-chromosomes should normally give a rod bivalent. In the case of chromosome pairs 2 and 3, an average of rather over one chiasma per arm is expected, or an average of about 2·5 chiasmata per chromosome. These figures are the minimum possible on the genetical data. There are, however, four chromatids, all of which may undergo crossing-over, two at a time at different points along their length; hence the probable maximum number of chiasmata is twice the minimum, namely, four minus for chromosome I and about five each for chromosomes II and III.

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[*] In *Primula sinensis* the range in length of the chromosomes, calculated from chiasma numbers, is 58·3 to 116·7 centimorgans; the SBGL chromosome is 111·6 centimorgans long (Darlington, 1931).

With regard to the small fourth chromosome pair, on the above basis it would apparently have a chiasma only about once in 50 times; in other words, the regular arrangement in *Drosophila* would be three bivalents and two univalents.

It is readily apparent, then, that the number of chiasmata possible upon the genetical data of *Drosophila* agrees roughly with the actual number seen in the bivalents of *Oenothera*, namely, a usual minimum of two and a maximum of four or rarely five. In diploid *Primula sinensis* there are 1 to 5 diplotene chiasmata, the mean being 3·5 per bivalent.

Summary and Conclusions.

Evidence in accord with the theory of parasynapsis and segmental interchange is presented for an *Oenothera* form, having normally a ring of six chromosomes and four ring bivalents. Parasynaptic pairing occurs between homologous segments of the ring-forming chromosomes and throughout the whole length of the two chromosomes of a bivalent, pairing usually commencing at the ends and proceeding towards the attachment constriction.

Interstitial chiasmata have been seen at late diplotene and are figured also at diakinesis and the heterotypic metaphase; they appear to be formed with a greater frequency towards the ends of the chromosomes. Terminalisation of the chiasmata occurs during diplotene and diakinesis; failure of formation of one diakinesis chiasma occurs in about 12 per cent., and failure of two chiasmata in rather less than 1 per cent. of nuclei.

The chromosomes are split longitudinally into two chromatids at some stage previous to diplotene; the bivalents thus have a tetrad structure, while the connections between chromosomes in the ring are double (this is clearly visible in favourable cases). Interlocking is a mechanical result of the pairing of the chromosomes commencing at the ends, and it occurs during synapsis.

The frequency of chiasmata is considered in general from a genetical basis, and it is shown that the long chromosomes of *Drosophila* probably have between two and five diplotene chiasmata each.

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The Reproductive Processes of certain Mammals. II.—*The Size of the Graafian Follicle at Ovulation.*

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I. *Introduction.*

It is generally recognised that the size of the mature Graafian follicle varies greatly from species to species, and that, roughly speaking, the size of the follicle at ovulation is greater in the larger mammals than in the smaller. So far as can be ascertained, however, no attempt has been made to examine this relationship accurately.

The present paper is primarily concerned with the correlation between the size of ripe follicle and the body weight of the species of animal, but a second point is also included. Brambell (1928) has shown that even in an animal such as the mouse, where the ripe follicle is comparatively small, the growth of the ovum is completed at an early stage, and that the subsequent growth of the follicle must, therefore, be concerned with some other function than the nutrition of the ovum. Brambell actually found that the growth of the ovum was completed before the formation of the antrum, *i.e.*, that the essential characteristic of the mammalian follicle only appears after the nutrition of the ovum has ceased to be an urgent function. It seemed of interest, therefore, to ascertain whether a similar state of affairs exists in the follicles of larger mammals, and if so, what relationship the size of the follicle, when the ovum is fully grown, bears in different species to the ultimate size of the follicle at ovulation.

II. *Methods.*

Size of the Mature Follicle.—In several species the follicle is known to undergo very rapid growth immediately before ovulation, and a similar condition probably occurs in all mammals ovulating spontaneously. It is thus necessary to obtain an extensive series of follicles approaching ovulation before the final mature size can be estimated with confidence, and at present accurate figures are available for comparatively few species. The figures given in Table I, column 5, have been obtained from the following sources: mouse, Brambell and Parkes (1927); rat, Long and Evans (1923); ferret, Robinson (1918), Hammond and Marshall (1930), Hill and Parkes (1930); rabbit, Walton and Hammond (1928); baboon, Zuckerman and Parkes (1931); pig, Corner (1921); cow, Hammond (1927). Other species might have been included if less accurate data had been accepted, especially by making the common assumption that the size of the ripe follicle is about four-fifths that of the fully developed corpus luteum (Corner, 1923); but, on the whole, it seemed preferable to confine the discussion to the above species, which form a fairly representative body-weight series.

Size of Ovum.—Among these seven species, data relating to the size of the follicle when the ovum becomes fully grown exist only for the mouse (Brambell, 1928) and the baboon (Zuckerman and Parkes, 1931). Comparable figures have therefore been obtained for the rat, ferret, rabbit, and pig by the methods used by Brambell. The ovary of the cow was found to be almost hopeless material for the purpose, on account of the extraordinary lack of small follicles. Even calves' ovaries were found to contain too few normal small follicles to provide material for the statistical methods involved. Considerable variation occurs in the size of the ova, but Brambell was able to show that in the mouse the growth of the follicle to resting size falls into two phases. In the first of these, both the ovum and follicle undergo coincident growth, whereas during the second and longer phase the ovum remains practically stationary in size, although the follicle is growing very considerably. In both of these two phases the size of the ovum bears a linear relation to the size of follicle, although in the second phase the increase in the size of the ovum is insignificant. The point of intersection of the two lines may thus be said to give the size of the fully grown ovum and the size of follicle at which its full development is attained.

Histological Technique.—Preparation of the rat, rabbit, and ferret material presented no difficulty. Cow and pig, and afterwards calf ovaries, were fixed at the abattoir as soon as possible after the animals were slaughtered. Bouin's fluid was employed as usual. Sections were cut at 7μ and stained in hæmatoxylin and eosin.

For measuring the size of the ova and follicles, camera lucida drawings were made of the largest section of the ovum, and the mean of two diameters at right angles found. Smaller follicles were measured in the same manner, but in the case of larger ones a third diameter, arrived at by counting the number of sections, was included for finding the mean. Care was taken to exclude all follicles not definitely normal.

Statistical Methods.—The points given by the measurements were then plotted for each species (with the exception of the cow, which, for reasons stated above, had to be omitted). It immediately became clear that in all four cases the initial growth of the ovum and follicle was characteristically of the type found by Brambell in the mouse, and could, presumably, be dealt with by the same statistical means. These methods are described in Brambell's paper, and need not be mentioned further here, except to say that the two phases are considered separately, the size of the ovum in each being dealt with as a simple straight-line regression on size of follicle. As a result, an equation is obtained for the size of follicle and ovum in each phase. The significance of the regressions was examined by Brambell, who found that, while the increase in size of ovum during the first phase was highly significant, the slight change in size of the ovum during the second phase was entirely insignificant. The results given here for the rat, ferret, rabbit, and pig are completely comparable with those found by Brambell for the mouse; in particular, graphical representation shows that the regressions are clearly of the same order. It has been thought unnecessary, therefore, to examine the significance of the regressions indicated by the further equations given in the present paper.

III.—*Size of Mature Follicle, and Body Weight of the Species.*

In Table I, column 5, are given the sizes of the ripe follicle in the seven species considered; these data are taken from the sources mentioned above. In column 1 are given the approximate weights of young adult females of each species. Fig. 1, line A, shows the result given by plotting size of ripe follicle

Table I.—Size of the Graafian Follicle at Various Stages of its Life-History.

Species.	1. Approximate weight of young adult female.	2. Diameter of ovum.	3. Diameter of follicle when ovum fully grown.	4. Diameter of follicle when antrum appears.	5. Diameter of follicle at ovulation.
	gm.	μ.	μ.	μ.	mm.
Mouse	2 × 10	70	125	200	0·55
Rat	1·2 × 10^2	63	160	200	0·90
Ferret	5 × 10^2	108	170	230	1·4
Rabbit	2 × 10^3	84	145	250	1·8
Baboon	1·2 × 10^4	83	180	310	6·0
Pig	5 × 10^4	76	300	400	8·0
Cow	4 × 10^5	—	—	—	15·0

against the weight of the animal, using a logarithmic scale in both directions. These seven points obviously give a close approximation to a straight line;

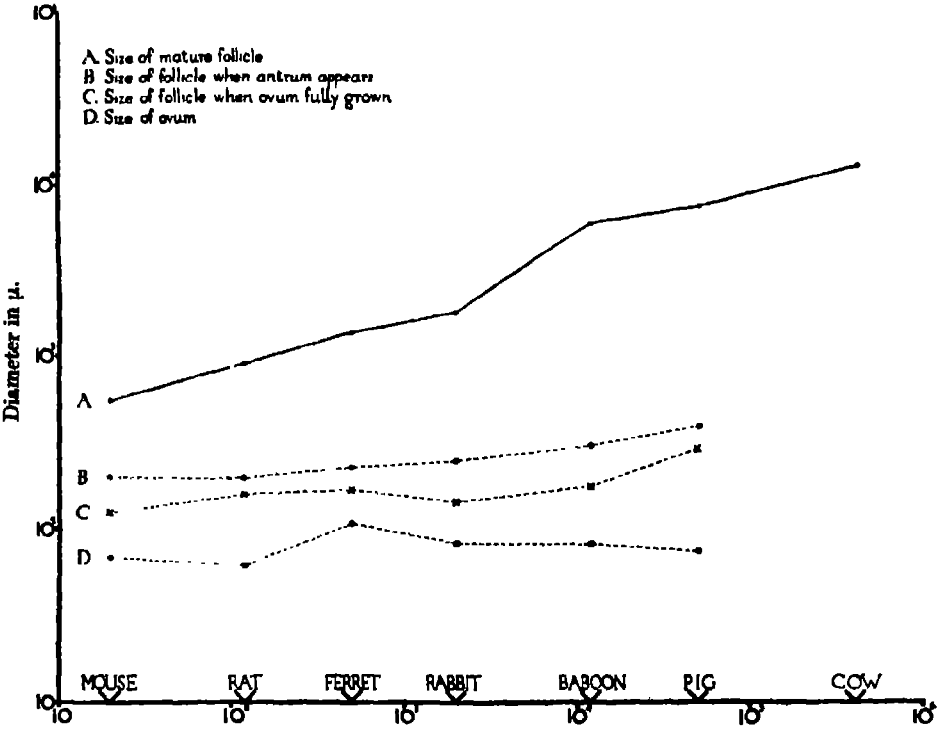

FIG. 1.—Size of ovum and follicle, and body weight in various species.

certainly there is no regular indication of a curve. At the moment one is hardly justified in putting a straight line through the points, but if further data, as they become available, indicate the same tendency, it will be possible to calculate the regression of size of ripe follicle on weight of the species. On the other hand, subsequent information may be quite contradictory; there is some evidence, for instance, that the mature follicle of the horse is far larger than would be suggested by the tendency of the points given on the graph.

Assuming, provisionally, that the points are falling about a straight line, certain deductions may be made. At the lower extreme small mammals, such as the Pipistrelle bat, should have a ripe follicle about 0·3 mm. in diameter, while at the upper extreme the ripe follicle of the whale should be about 8 cm. in diameter. Both of these estimates agree fairly well with what can be ascertained about the follicles in these mammals. It is highly significant that the sizes of the ripe follicles of the two monotocous species (baboon and cow) fall so closely on the line given by the polytocous species. In other words, the size at which the follicle ovulates is not affected by the number produced in the ovary at one time. Another point of interest is the fact that the seven species dealt with cover a wide zoological range: three Rodentia, one Carnivora, two Ungulata and one Primate.

In fig. 1 the diameter of the follicle is plotted against the weight of the animal. The proper course would obviously be to plot the volume of the follicle, so that some approximation to mass of follicle against mass of animal would be achieved. Actually, however, retaining the double log. scale, the conversion of the diameters to volumes would not alter the straight line relationship, although the slope of the line would, of course, be much steeper.* Conversion to volumes would, however, make it difficult or impossible to show the other lines (size of ovum, etc.) on the same diagram,† and the diameter measurement is, therefore, retained. The linear relation of the diameter on double log. paper implies that on an ordinary scale they would fall on a gradually flattening curve, but it is clearly necessary that more material should be available before further discussion is of value.

IV.—*Initial Growth of the Ovum and Follicle.*

Whatever the precise relationship may be between the size of the mature follicle and the weight of the species of animal, it is necessary to consider what function can be served by the relatively enormous follicles of the larger mammals. Brambell showed conclusively that even in the mouse the later development of the follicle was not connected with the nutritive requirements of the ovum, and a similar condition has also been found in the baboon. In figs. 2–5 new data are presented for four other species; *i.e.*, rat, ferret, rabbit and pig. In all these instances the same two characteristic phases occur in the early life history of the ovum and follicle; (*a*) an early phase during which the ovum grows, and the follicular epithelium, though solid, proliferates (line *a* on the diagrams); (*b*) a later phase, during which the size of ovum is practically stationary, though the follicle continues to develop, obtains an antrum and finally proceeds to maturity (line *b* on the diagrams). A summary of the data shown in these diagrams, together with the approximate size of the follicle when the antrum appears, is given in Table I, columns 2, 3, 4 and Table II.

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* Cubes plotted against their roots fall on a straight line on double logarithmic paper.

† In the cow, the volume of the ovum is about 1/3-millionth of the volume of the ripe follicle. To cover a range of this magnitude (even on a log. scale) would necessitate a diagram so large that all detail would be lost on reproduction.

Table II.—Equations for the Size of Ovum (y) in relation to Size of Follicle (x) in the Rat, Ferret, Rabbit and Pig.

Species.	Equation.	
	Phase *a*.	Phase *b*.
Rat	$y = 17 \cdot 73 + 0 \cdot 2861\, x$	$y = 63 \cdot 11 + 0 \cdot 0017\, x$
Ferret	$y = 9 \cdot 22 + 0 \cdot 5848\, x$	$y = 108 \cdot 37 - 0 \cdot 0030\, x$
Rabbit	$y = 15 \cdot 13 + 0 \cdot 4730\, x$	$y = 82 \cdot 38 + 0 \cdot 0105\, x$
Pig	$y = 35 \cdot 22 + 0 \cdot 1382\, x$	$y = 74 \cdot 95 + 0 \cdot 0049\, x$

The corresponding data for the mouse and baboon are recorded elsewhere (Brambell, 1928 ; Zuckerman and Parkes, 1931).

When the values given in Table I, columns 2, 3 and 4, are plotted according to weight of species, for comparison with the size of ripe follicle, it is seen that :—

(*a*) The actual size attained by the ovum (fig. 1, line D) has no correlation with the body weight of the species or with the ultimate size attained by the follicle. Nor does the size of the fully grown ovum determine the size of the follicle when the ovum becomes full size. The largest egg in this series is actually that of the ferret.

(*b*) The size of follicle when the ovum becomes fully grown (fig. 1, line C), shows some species variation, a slight increase being found in the larger mammals. Thus, in the pig the follicle is about 300μ in diameter when the ovum becomes fully grown, compared with a corresponding figure of 125μ for the mouse. On the other hand, the diameter of the ripe follicle of the pig is about 15 times that of the mouse, so that the size of the ripe follicle is not conditioned by the requirements of the ovum.

(*c*) The size of the follicle when the antrum appears is rather variable, even in one ovary, but the figures given in Table I represent in each case the diameter of the smallest follicle observed with a definite antrum. In all cases (fig. 1, line B), the size is just above that at which the ovum becomes fully grown. A slight increase in the larger species is also found. It is difficult to see why the follicles of the larger mammals, not having larger ova, should be regularly, though slightly, larger at the time when (i) the ovum is fully grown, and (ii) the antrum appears.

If one accepts the usual assumption that the follicles of Eutherian mammals ultimately develop an antrum, then in these animals the size of the mature follicle cannot be less than the size at which the antrum appears. If the points constituting lines A and B in fig. 1 are assumed to fall about straight lines, then it is clear that the two lines would intersect if projected backwards. The place of intersection would represent the point at which the follicle was mature as soon as the antrum appeared, *i.e.*, would indicate the smallest follicle compatible with the definition of a mammalian follicle. The intersection of the lines would take place over a point on the base line representing a body weight of 2–3 gm. This weight therefore represents the smallest mammal which could have a true Graafian follicle. Actually, the smallest known mammals appear to be *Microsorex* s/s of North America, weighing about 2·5 gm.

Characteristics of the Initial Growth of the Ovum and Follicle in Various Species.—Various conclusions relating to the comparative features of the initial growth of the ovum and follicle may be drawn from figs. 2–5, together with the data for the mouse and the baboon already mentioned. The (*a*) lines in figs. 2–5, if projected, would in all cases meet the side line; in other words, the ovum, a part of the follicle, would have a definite size before the follicle existed. Thus, the early growth of the ovum can not be represented by an extension of the *a* line. The discrepancy is due to the fact that during the initial growth of the ovum, there is no measurable amount of epithelium round it, *i.e.*, the size of the ovum is the same as the total size of the follicle. The growth of the ovum, under these conditions, is shown in figs. 2–5 by the dotted line. The point of intersection of this dotted line with the *a* line gives the point at which the size of the whole follicle begins to get larger than that of the ovum, *i.e.*, the

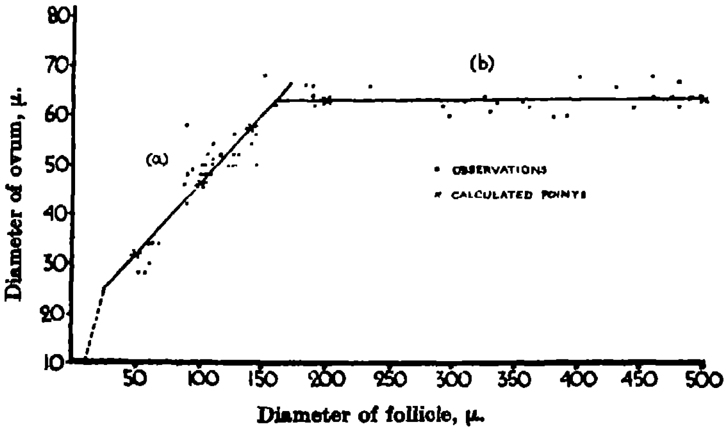

FIG. 2.—Initial growth of the ovum and follicle in the rat.

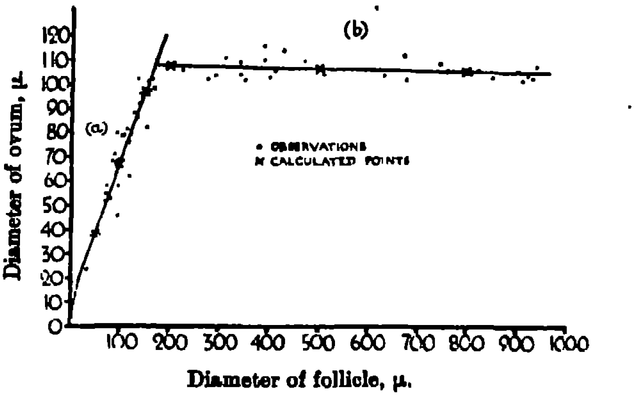

FIG. 3.—Initial growth of the ovum and follicle in the ferret.

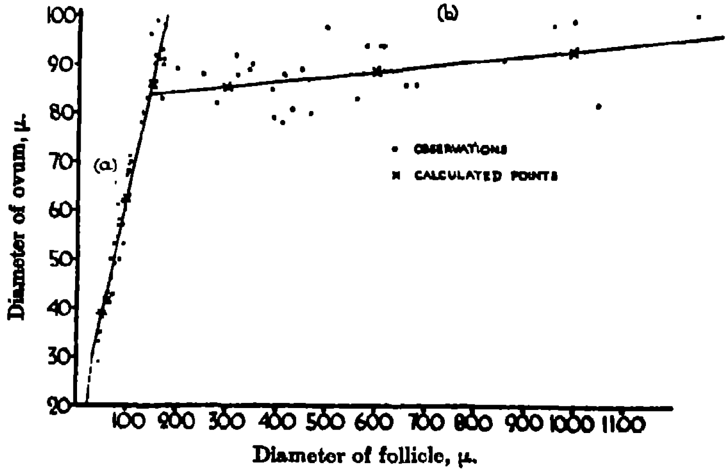

FIG. 4.—Initial growth of the ovum and follicle in the rabbit.

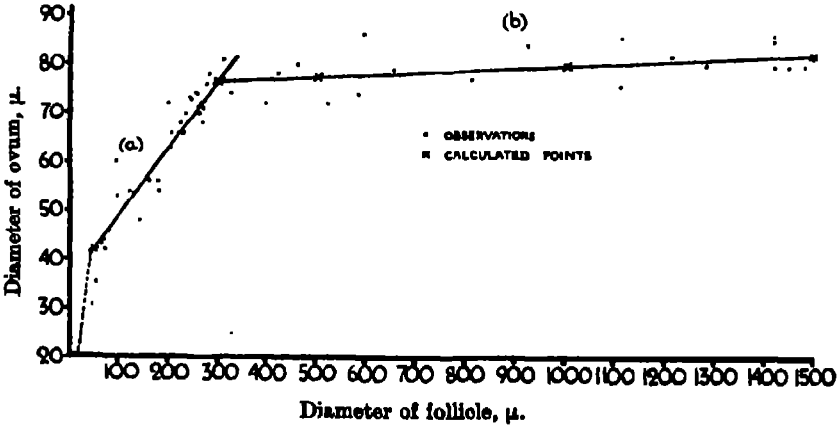

FIG. 5.—Initial growth of the ovum and follicle in the pig.

point at which the ovum begins to acquire a measurable amount of follicular epithelium. For ease of comparison, the *a* lines of figs. 2–5, together with those of the mouse and baboon, are reproduced, all to the same scale, in fig. 6, the dotted line again representing the initial growth of the ovum without epithelium. From this diagram it is evident that the ovum of the mouse begins to acquire appreciable epithelium very early, at about 13μ, while the pig ovum becomes much larger, a little over 40μ, before reaching a similar stage. The ova of the rabbit and the rat are about 30μ, and of the baboon and ferret about 25μ, before measurable epithelium appears.

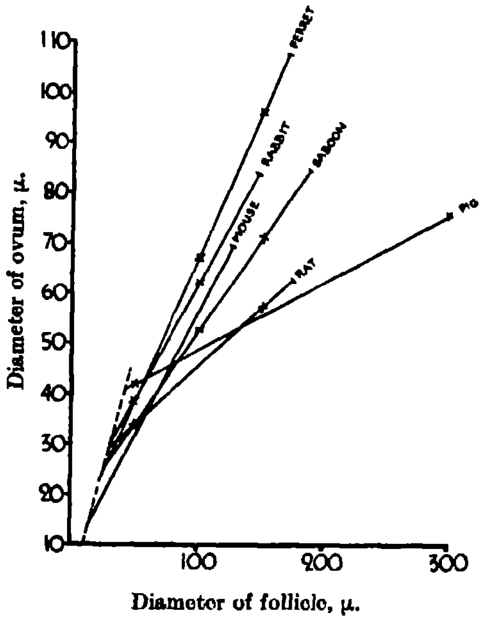

Fig. 6.—Comparative growth of the ovum in various species of mammals.

Measured in terms of the size of follicle, the ovum of the ferret grows fastest and the pig slowest, the mouse, rabbit, baboon and rat falling in that order intermediately. This order differs, of course, from that given by "size of follicle when ovum fully grown," which is a product of (*a*) rate of growth of ovum, and (*b*) final size of ovum.

It is not possible to estimate directly the actual time taken by the follicle to reach its various phases of growth, but the comparative paucity, in all six species, of follicles immediately above the size at which the ovum becomes fully grown, suggests that a period of unusually rapid growth occurs at this time.

V.—*Discussion.*

The facts recorded above indicate a very general applicability of Brambell's conclusion for the mouse, that the growth of the ovum is completed very early in the life-history of the follicle, and that the whole of the subsequent growth must therefore take place to facilitate some other function than the actual nutrition of the ovum. In the larger mammals, in spite of the fact that the follicle is rather larger by the time the ovum has reached full size, the subsequent growth of the follicle is proportionately much greater than in the mouse. The ratios of "size of follicle when ovum fully grown" to size of mature follicle are as follows:—mouse, 1 : 4·4; rat, 1 : 5·6; ferret, 1 : 8·2; rabbit, 1 : 12·4; baboon, 1 : 33·3; pig, 1 : 26·6. Brambell's conclusion is thus not merely confirmed by these data, it is greatly strengthened.

Two inter-related problems arise out of this investigation:—(*a*) Brambell's original problem, accentuated by the additional information for other species, as to the purpose of the second phase of follicular growth, and (*b*) why this second phase should be so exaggerated in the larger mammals. Certain considerations relating to the first have been discussed by Brambell, but some additional points raised by the extension of the data and relevant to the second problem, may be mentioned:—

(*a*) The actual mechanical pressure necessary to cause rupture can clearly be attained in quite small follicles (*e.g.* mouse), so from this point of view larger follicles (*e.g.* the cow) would not be required. It is, however, necessary to consider the mechanical problem of attaining a position from which ovulation can take place. In some animals the maturing follicle actually comes to the surface of the ovary partly as a result of its distention. In all cases the distention presumably attenuates the peripheral ovarian tissues to the extent required to permit of their being ruptured at ovulation. In animals such as the baboon, with a thick fibrous tunica, this preliminary attenuation must be very necessary. In all mammals possessing large ovaries, very considerable difficulty would be experienced by the follicle in reaching a strategic position for ovulation, without the distention occurring during maturation.

(*b*) It is not probable that the larger follicle found in the larger mammals contributes much to the greater œstrin production required. In an animal like the baboon, the actual total amount of granulosa present in the follicle increases comparatively little during maturation. The increased size of the follicle is due mainly to the increase of liquor folliculi. The same is almost certainly true of the other large mammals. In any case, the follicular granulosa is not the only possible source of œstrin in the ovary. On the other hand, it is quite reasonable to suppose that the larger mammals require a much larger corpus luteum than the small mammals, and a large cavity formed by the ruptured follicle offers the best facilities for the rapid development of a large corpus luteum.

(*c*) It was pointed out by Brambell that the liquor folliculi liberated at ovulation must play some part in washing the ovum into the Fallopian tube. In the large mammals, lacking the complete ovarian capsule found in the mouse, the ovum may be dependent upon the liquor folliculi for being washed into and down the mouth of the tube, and the large liquor content of the mature follicle may be essential from this point alone.

It is a pleasure to express my thanks to Professor F. W. Rogers Brambell for his advice and assistance.

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VI. *Summary*.

(1) This paper deals with the size of the mature Graafian follicle in various species and its correlation with the body weight of the species. The initial growth of the ovum and follicle in the rat, ferret, rabbit and pig is also dealt with (following up Brambell's work on the mouse).

(2) The relation between the size of mature follicle and the body weight of the animal is examined in the mouse, rat, ferret, rabbit, baboon, pig and cow. In this series, the size of ripe follicle varies from 0·55 mm. in the mouse to 15 mm. in the cow, while the corresponding body weights range from 2×10 gm. to 4×10^5 gm. The diameter of the ripe follicle plotted on double log. paper against the weight of the species falls on a close approximation to a straight line.

(3) The size of the follicle, when the ovum has become fully grown, is slightly greater in the larger mammals than in the smaller, but in all cases the growth of the ovum is completed at a very early stage in the life history of the follicle. The ratio of the size of the follicle when the ovum is fully grown to the size of mature follicle decreases considerably in the larger mammals, being: mouse, 1 : 4·4; rat, 1 : 5·6; ferret, 1 : 8·2; rabbit, 1 : 12·4; baboon, 1 : 33·3; pig, 1 : 26·6.

(4) The size of the mature follicle is thus not conditioned by the requirements of the ovum. The significance of the large size of the mature follicle, especially in the larger mammals, is discussed.

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The Functions of the Corpus Luteum. V.—Changes in the Sterile Horn during Pregnancy, and their Relation to Changes in the Corpus Luteum.

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[Plates 19–21.]

I. *Introduction.*

Evidence as to the precise rôle of the corpus luteum during pregnancy is contradictory, especially when different species are compared. Most observers[*] agree that the presence of the corpus luteum is necessary during the early stages of gestation. During the second half of pregnancy, however, the removal of the corpus luteum in certain animals does not lead to immediate abortion. In the guinea-pig Loeb and Hesselberg (1917), Herrick (1928) and Courrier, Kehl and Raynaud (1929) have found that pregnancy may continue for some weeks after double ovariectomy, and Ash-Upmark (1926) has listed a large number of clinical cases in which ovariectomy in women has not disturbed pregnancy. The evidence for the rabbit (summarised by Hammond, 1925), is slightly contradictory, but tends to show that the corpus luteum is necessary during the whole of pregnancy, although this was not the conclusion of Niskoubina (1909). In the rat it appears that double ovariectomy towards the end of pregnancy does not always result in its premature termination (Marshall and Jolly, 1906). More definite information is available for the mouse (Harris, 1927; Parkes, 1928), where the presence of the corpus luteum is essential during the whole of gestation.

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[*] No attempt is made here even to summarise the mass of experimental work on this subject; for further references see Marshall (1922) and Parkes (1929)

A review of this evidence suggests that there are marked species differences, the corpus luteum being necessary during the whole of pregnancy in some mammals, but not in all. The experiments of Allen and Corner (1929) make it evident that a true hormone effect is involved, though apparently they have not yet determined the required duration of luteal activity in pregnancy.

Another line of investigation is provided by histological examination of the corpora lutea at various stages of pregnancy to ascertain the time of onset of degenerative changes. In most corpora lutea definite regressive histological changes can be distinguished at parturition, or, in the unmated animal, at the time of the next ovulation. The regression occurs in certain animals some time before the luteal phase of the cycle is definitely terminated (references in Deanesly, 1930) and it might be inferred that in these cases corpus luteum activity had ceased. The difficulty lies in assessing the functional significance of the degenerative histological changes in their initial stages; they may indicate complete cessation or merely slowing down of the secretory activity of the lutein cells. In either case the effect on other organs of the body, such as the uterus, will depend on the latent period, the threshold value and the possibility of storage of the luteal hormone.

Progress in the estimation of the functional activity of the corpus luteum from its histological appearance has also been retarded hitherto by the absence of clearly defined criteria of regression and by the varying histological methods used. As regards the former it has been usual to consider an accumulation of histologically stainable fats and lipoids in the lutein cells (especially those shown after osmic acid fixation), as a mark of regression, but a study of corpora lutea belonging to different species suggests that this view needs qualification. In some animals fats and lipoids fill the lutein cells during the early stages of pregnancy and have almost disappeared from them at the time of parturition. The contradiction is only an apparent one; in all corpora lutea of pregnancy so far examined the fatty cell inclusions fluctuate for a while, then reach a well-marked maximum and finally decline. The evidence indicates that the maximum fatty phase coincides approximately with the regression of the corpus luteum, but it may occur half way through pregnancy, as in the rabbit, or shortly after parturition, as in the mouse and guinea pig. Owing to the variation between different species in the amount of lutein cell fat reacting to osmic acid during the early stages of pregnancy, the nature of the fat granulation is a better index to the functional value of the corpus luteum than its quantity; in older corpora lutea the granulation becomes more coarse and irregular. A definite and reliable characteristic of corpus luteum regression is, of course, the slowing down of the vascularisation and the shrinkage of the lutein cells and their nuclei, but where this process begins before parturition it is gradual, and, allowing for normal variation among the corpora lutea, its exact time of onset cannot always be clearly distinguished. The degeneration of the corpus luteum at the end of pregnancy has been held to be a factor producing parturition, but it is possible that the same over-ruling cause brings about both results.

A biochemical study of the relation of the lipoids to physiological activity in the corpus luteum of the sow has been carried out by Bloor, Okey and Corner (1930). Goormaghtigh (1927) has discussed the histochemistry of lutein cell fats and lipoids and their relation to the secretory product. The present work includes an inquiry into the functional significance of the histological changes which take place in the corpus luteum of pregnancy, and not into their actual nature.

Corpus luteum function may be investigated by a third method, recently elaborated—the study of the changes during pregnancy in the endometrium of a uterine horn rendered sterile by salpingectomy or unilateral ovariectomy. It is generally agreed that the corpus luteum is responsible for the early progestational growth in the endometrium. The subsequent changes in the endometrium in a region undisturbed by fœtuses, but under the same hormonal influences as the pregnant part of the uterus, may be considered diagnostic of the activity and duration of the luteal secretion, provided that the endometrium is capable of prolonged response. Courrier and Kehl (1929) and Knaus (1930) have shown that in the rabbit the progestational condition of the sterile horn does not persist right through pregnancy. Degeneration sets in at about

16 days p.c., namely, at about the same time as in pseudo-pregnancy, though the actual degeneration is slightly slower in the sterile horn. On this evidence, therefore, the rabbit corpus luteum would appear to cease functioning about half-way through pregnancy.

Courrier and Kehl (1930, *a*) have claimed, however, that the endometrium was incapable of maintaining the progestational condition for more than 14 to 16 days, even when subjected to the action of relays of artificially produced corpora lutea. These results, if correct, would invalidate the evidence as to the duration of lutein activity obtained from the sterile horn, but they are in contradiction to those of a similar experiment already reported (Parkes, 1929, *b*). Further data, to be recorded elsewhere, have shown that under certain conditions the duration of the progestational condition of the sterile horn may be prolonged by the presence of a second set of corpora lutea, but that the reaction is not constant.

Less is known of the changes occurring during pregnancy in the sterile horn of other animals, though some information is available for the ferret (Hammond and Marshall, 1930) and the marsupial Bettongia (Flynn, 1930). For the work described in this paper the mouse, rat and guinea-pig were studied in addition to the rabbit, and an attempt was made to correlate the uterine evidence of duration of luteal activity with the histological appearance of the corpora lutea.

II. *Material and Technique.*

Unilateral sterility of the uterus was produced as a routine by left salpingectomy, the animals being mated as soon as practicable after the operation. The uteri were fixed in Bouin's fluid, and the sections stained in Ehrlich's hæmatoxylin. One or two specimens of sterile cornua in mice were obtained from the unilateral X-irradiations previously described (Parkes, 1928).

The histological technique for the ovaries and corpora lutea has been described elsewhere (Deanesly, 1930). Flemming's, Ciaccio's and Bouin's fixatives were used, and sections cut at 7 μ. The first two fixatives preserved fat and lipoid cell inclusions; the osmic acid in Flemming is said to blacken olein and oleic acid and mixtures containing a sufficient amount of these substances, while, with Ciaccio's method for lipoids, mixtures of cholesterol and neutral fats can be stained, and also certain phosphatids including lecithins.

An expression for the size of the corpora lutea was obtained, as before, by measuring two diameters at right angles to each other across the largest section of each corpus luteum. The measurements for mice (Deanesly, 1930) represent the average of several corpora lutea belonging to a single ovulation, but those for the rabbit and guinea-pig represent the largest corpora lutea measured from each animal; as such they can only have an approximate value, owing to normal variation. An apparent increase or decrease in corpus luteum diameter was checked, however, by direct comparison of the lutein cells at the two stages. In older corpora lutea there may be a certain amount of flattening so that the mean diameter may be less accurate in proportion to the true size of the corpus luteum than in recent ones.

III. *The Rabbit.*

Changes in the Sterile Horn during Pregnancy.—Seven specimens are available of the sterile horn of the rabbit, ranging from the sixteenth to the twenty-eighth day of pregnancy. During this time the condition of the endometrium is exactly comparable to that described by Courrier and Kehl (1930, *a*) and Knaus (1930), and also found by Hammond. At sixteen days the mucosa still shows the typical pseudo-pregnant condition, but signs of degeneration are beginning to appear. At 19 to 20 days pregnant the stroma is more compact and the glands are less obvious, but no débris is seen in the lumen. Both specimens taken at 23 days pregnant show a greater departure from the pseudo-pregnant condition, having a more compact stroma and less extensive glands. UPR 5 shows an abundance of multinucleate giant cells or syncytial masses free in the lumen; these are clearly conglomeration products of the cells which may be observed sloughing off from the epithelium (Plate 21, figs. 10 and 11). In UPR 1, however, there appears to be neither the typical pseudo-pregnant growth nor the desquamation of cells into the lumen which characterises the later stages of the breakdown in the sterile horn. A study of this specimen in conjunction with the corresponding corpus luteum suggests that the degeneration of the endometrium has already been completed in this animal. In other rabbits the process is continuing at 26 to 28 days pregnant. The stroma becomes more compact as the glands shrink and degenerate, the superficial layers being removed by sloughing. There is no desquamation of strips of tissue, but cells break away singly or in small groups. At 26 to 28 days pregnant very large giant cells are found in the lumen, up to 40 μ in diameter. The necrotic tissue surrounding the lumen can be plainly distinguished; apparently all is cleared away before the regeneration process preceding the next œstrus begins. At 28 days the sloughing is complete in some areas. No hæmorrhage has been observed in the sterile horn at any stage of the breakdown of the endometrium; the folds of the latter are often very vascular, but no signs of hæmatomata in the tissues or of bleeding into the lumen were found.

Table I.—Changes in the Sterile Horn of the Pregnant Rabbit.

No. of rabbit.	Days pregnant.	Diameter of corpus luteum. mm.	Condition of sterile horn.
UPR 3	16	2·78	Pregestational endometrium showing signs of degeneration.
UPR 6	19	—	Stroma more compact; glands decreasing in size and complexity.
UPR 4	20	2·43	Similar to UPR 6.
UPR 1	23	2·80	Glands smaller; no cells sloughing off into lumen; degeneration may be completed.
UPR 5	23	2·90	Large number of cells sloughing off into lumen; no apparent hæmorrhage.
UPR 8	25	2·41	—
UPR 2	26	2·65	Stroma compact; much sloughing.
UPR 7	28	—	Stroma compact and very vascular. Sloughing complete in places and pro-œstrous regeneration of the epithelium beginning.

Courrier and Kehl (1930, *b*) found that although deciduomata could be induced in the sterile horn of the rabbit at 5, 6 and 8 days pregnant, they could not be induced at 12, 15 and 20 days pregnant, as might be expected from the degenerating condition of the endometrium.

The Histology of the Corpus Luteum.—Table I gives some idea of the size of older corpora lutea in the rabbit. As previous workers (Niskoubina, 1909, etc.) have observed, there is a change in the vascularisation of the corpus luteum soon after mid-pregnancy; it no longer appears pink on the surface of the ovary, but tends to become pale and partially collapsed. The change in colour may be due in part to a thickening of the stroma and germinal epithelium over the surface. Allowing for normal variation in the size of the corpora lutea and for a slight decrease attributable to the slowing down of the vascularisation, it seems that they persist almost at their maximum until just before parturition. Only UPR 2 (26 days) and SM 10, a late undated pregnancy, showed clear indications of cell shrinkage.

At 8 days after copulation, when the egg is becoming implanted, the lutein cells have nearly reached their full size, and the corpus luteum diameter in SM 9 is 2·57 mm. Sections through it show a uniform distribution of osmicated fat granules; lipoids are moderately plentiful in the outer part of the corpus luteum, but less deeply staining in the inner region.

At nearly 14 days after copulation the corpus luteum measures 2·9 mm. It has not yet declined from the maximum size, but it shows a striking difference from the preceding ones after Flemming fixation; the fats blackening with osmic acid are now restricted to a wide exterior zone and are absent from the more deeply lying parts of the corpus luteum (Plate 20, fig. 7). The lipoids, stained by Ciaccio's method, show a similar distribution, but the transition between outer and inner regions is gradual, and there is no conspicuous change from the 8-day stage.

The gradual restriction of osmicated fatty substance to a decreasing peripheral zone could be traced in all but one of the seven later pregnancy corpora lutea examined; the exception was UPR 5, where such fats persisted throughout the corpus luteum. They tended to become progressively heavier and more coarsely and irregularly granulated in the later pregnancies. The series is not perfectly regular since UPR 8 (25 days pregnant) still retains a larger amount of osmicated fat than some earlier stages, but the general tendency is unmistakable. A similar peripheral zoning of the darker staining lipoids can be seen using Ciaccio's method. Frozen sections through corpora lutea showed that although fat was present in all the cells, yet darker staining substances occurred in the outer rim, so that the latter cannot be regarded as a histological artifact.

It may be provisionally suggested that this gradual restriction of certain fatty substances to the edge of the corpus luteum is connected with the decrease of the vascular supply, although, from sections, it cannot be clearly determined whether the blood supply is longest maintained among the exterior cells, which apparently continue to elaborate or store the deeply staining fats and lipoids. The heavier deposits of these substances in the zone where they persist suggest that the rate of excretion has fallen off. In UPR 5 (23 days pregnant) the corpora lutea were large and apparently well vascularised, and heavy deposits of osmicated fat were present in all the cells (Plate 20, fig. 8). Since cell sloughing is in progress in the sterile horn of the corresponding uterus, it may be inferred that the functional activity of the corpus luteum has waned, and that the abundance of fats, in contrast to other corpora lutea of a similar age, is due to absence of excretion or a difference in their composition, and not to hyperactivity of the lutein cells.

In the rabbit, therefore, where the lutein cells at their functional period contain osmicated fats, the histological changes suggest that the secretory activity of the corpus luteum declines from about the middle of pregnancy, although a reduced activity may continue during the last 2 weeks, the rate of regression varying somewhat in different animals. A similar histological change appears to take place in the corpus luteum of pregnancy of the cat, but insufficient material is available to show when it occurs.

A comparison of the condition of the corpora lutea with that of the sterile uterine cornua of the five rabbits in which both were studied, UPR 1 to 5, shows a fairly definite correspondence between the two. The progestational endometrium presents signs of degeneration in UPR 3 where osmicated fat is restricted to a broad outer zone of the corpus luteum, and further degeneration in UPR 4, when the fatty area of the section has again contracted. In UPR 1, 2 and 4 the fatty area is of about the same extent, but the granulation is most regular in UPR 4 and least so in UPR 1. It seems probable that degeneration had occurred earliest in the latter, where the uterine glands were small and the sloughing process probably complete.

Corpora lutea histologically similar to those of the last half of pregnancy were found in an ovary from a non-pregnant rabbit 18 hours after copulation, ruptured follicles being also present. From this it may be deduced that the former type of corpora lutea are no longer functional.

IV. *The Mouse.*

Changes in the Sterile Horn during Pregnancy.—In the rabbit, it is clear that the changes in the sterile horn during the second half of pregnancy are associated merely with the destruction of the previous progestational growth of the endometrium. A different condition is found in the mouse; during the second half of pregnancy the endometrium takes on a new character. For the first 10 days of pregnancy the development of the sterile horn is similar to that of the uterus in pseudo-pregnancy, following sterile copulation; moreover, this phase lasts about the same time in the two cases. Regression and return to the pro-œstrous condition then supervenes immediately in the pseudo-pregnant mouse, but in the sterile horn of the pregnant animal an entirely fresh phase of development has begun by the 13th day. This consists in an increase of the stromal glands, till they may be as large as 280 μ in diameter; where many glands occur together the uterine lumen may be partially blocked. The epithelial lining of the glands appeared very stretched and attenuated, and in some instances almost obliterated; in the sections examined the absence of any obvious secretion in the glands made it difficult to attribute the distention to any particular cause. The condition might almost have been considered pathological had not glands of this type been present in the sterile cornua of all five mice killed between 13 days pregnant and term. The material available is summarised in Table II.

Table II.—Changes in the Sterile Horn and Growth of the Corpus Luteum in the Pregnant Mouse.

No. of mouse.	Days pregnant.	Corpus luteum diameter. µ	Condition of sterile horn.
UCP 5	6	652	As in similar stage of pseudo-pregnancy.
SM 62	8	670*	,, ,, ,,
UCP 9	10	810*	,, ,, ,,
UCP 7	13	976*	Excessive development of glands beginning.
UCP 8	16	960*	Large glands well established.
UCP 2	17	—	Large glands ; stroma very congested.
UCP 1	18	982*	Large glands ; hæmorrhage into stroma.
SM 47	18	—	,, ,, ,,
UCP 6	0 day post-partum	864*	Glands and stroma undergoing degeneration ; no hæmorrhage into lumen.
UCP 3	1 day post-partum	732	As UCP 6.
UCP 4	2 days post-partum	816*	Uterus returning to normal.

* Mean diameter of pregnancy corpora lutea in other ovaries at the same stage.

By the 17th day of pregnancy the glands in the mucosa of the sterile horn are relatively enormous, and the stroma intensely congested (Plate 19, fig. 2). At 18 days the glands show little change, but numerous red blood corpuscles are present in the stroma, especially under the epithelium ; in places they are so numerous as to constitute hæmatomata (Plate 21, fig. 12). The process is a greatly exaggerated replica of that terminating pseudo-pregnancy in the mouse when, owing to congestion, slight bleeding may take place into the stroma. In many ways the condition resembles that of the pre-menstrual Primate uterus, but although a certain amount of tissue destruction takes place during the subsequent return of the mouse endometrium to normal, only very slight hæmorrhage into the lumen was observed. To judge from the present material the reaction of the uterus during the immediate post-partum œstrus is very slight.

If it be assumed for the present, in the absence of contrary evidence, that the secondary uterine changes during pregnancy are not due to fœtal or placental activity, then, in the mouse they suggest most strongly that luteal activity is continued until about the 17th day—a conclusion in complete agreement with previous work on removal of the corpus luteum during pregnancy (Parkes, 1928).

The Corpus Luteum of Pregnancy and Lactation.—The mouse corpus luteum has been described in detail elsewhere (Deanesly, 1930). During the first

8 or 9 days it is no larger than the corpus luteum of pseudo-pregnancy, its mean diameter being between 600 μ and 700 μ. Between the 9th and 13th days the lutein cells undergo great enlargement and the mean diameter of the corpus luteum rises to 900 μ–1000 μ and remains at that level until parturition. The fat and lipoid content of the lutein cells fluctuates during pregnancy, but is scanty in comparison with that of other rodents. After parturition the fats increase and the cells shrink and become disorganised.

The histological indications of corpus luteum function during pregnancy are in close agreement with the experimental evidence, since (1) during the first part of pregnancy the corpus luteum does not appear larger or more active than that of pseudo-pregnancy and the extent of development of the uterine glands is approximately the same; (2) during mid-pregnancy, at a time when the embryos are growing rapidly, the corpus luteum enlarges and its enlargement is followed by a new phase of growth of the uterine glands; and (3) the corpus luteum shows no histological signs of degeneration until just before parturition and experiment has shown that it is necessary for the maintenance of pregnancy up to this time. Corpora lutea of lactation were examined in 11 mice and have been described elsewhere; six of these mice were killed between 12 and 39 days post-partum. In the last two of these a fresh ovulation had occurred, but in the other four the follicles showed no sign of pro-œstrous enlargement. In these ovaries the corpora lutea of lactation were all in early or late stages of histological degeneration, and it was concluded that they were not functionally active.

The Uterine Changes during Lactation.—It is well known that, following the œstrus which occurs about 18 hours after parturition, there is no further œstrus in the lactating mouse for at least 3 weeks (Parkes, 1926; Crewe and Mirskaia, 1930). Removal of the young at any time during lactation results in the appearance of œstrus within 2 to 4 days. These facts are usually attributed to the influence of suckling on the corpus luteum formed at the post-partum œstrus; the latter is held to persist and function during lactation and atrophy at about 3 weeks post-partum, or when the young are prematurely removed. Although the corpus luteum of lactation may long persist, evidence that it is actively functioning during the whole of this time is not strong. During the first part of lactation there is good reason to suppose that it is as functional as during pseudo-pregnancy or the first part of pregnancy (Parkes, 1929, *a*). There is little or no evidence, however, that during the second half of lactation it functions in any capacity.

The work described above on the sterile cornu of the pregnant mouse shows clearly the response of the endometrium to luteal activity prolonged past the ordinary duration of pseudo-pregnancy. No such response is found during the second half of lactation. Our observations on thirteen animals show quite definitely that while there is some approach to the pseudo-pregnant condition during most of lactation, the uterus does not assume the extraordinary glandular condition found in the sterile horn in the second half of pregnancy. Only one animal of the six killed after the 12th day of lactation shows any approach to the "super-pseudo-pregnant" condition; this was killed on the 24th day of lactation. The others, as well as the seven obtained during the earlier part of lactation, exhibit only a poorly developed pseudo-pregnant condition. The size of the uteri was much below normal.

The condition of the uterus indicates clearly that the luteal activity during lactation is different in kind or duration from that in pregnancy. It is doubtful to what extent, if any, the persistent corpus luteum in the mouse is functional during the second half of lactation. It may become necessary to postulate that the absence of ovulation in this second period is due not to the persistence of a luteal phase but to a "lactation anœstrus," such as is found in the rabbit, ferret and many Primates (Zuckerman, 1931).

V. *The Rat.*

Changes in the Sterile Horn during Pregnancy.—In view of the striking similarity in the reproductive processes of the two animals, it was thought desirable to ascertain whether the extraordinary development of the sterile horn in the mouse had any parallel in the rat. Material was obtained from the latter animal at 14, 19 and 21 days pregnant. No very obvious differences between the three specimens were seen; there was no sign whatever of the strange glandular condition found in the mouse. The 14 and 19-day stages had a large uterine lumen with convoluted outline and thick epithelium with numerous small, almost solid glands—a condition reminiscent of that of pseudo-pregnancy. The 21-day stage had a shallower epithelium and fewer of the small glands.

The material available is insufficient to show the exact nature of the changes occurring in the sterile horn of the rat during the second half of pregnancy and their relation to the pseudo-pregnant condition, but it is evident that they differ greatly from those in the mouse.

The Corpus Luteum of Pregnancy.—The corresponding rat ovaries were inadequate in number for a study of the corpus luteum of pregnancy in this animal. According to Long and Evans' memoir (1922) it is similar in type to that of the mouse, and in section shows an accumulation of osmicated fat just before and after parturition. It continues to grow after the 10th day of pregnancy; in the ovary examined it had not reached its full size at 14 days pregnant, its diameter being 1·52 mm. In the 19 and 21-day stages when the diameters measured 2 mm. and 1·67 mm. respectively, the corpus luteum in section appeared to be losing fat, so that possibly it ceases to function slightly earlier in pregnancy than that of the mouse.

VI. *The Guinea-pig.*

Changes in the Sterile Horn during Pregnancy.—It was thought that examination of the changes in the sterile horn of the guinea-pig would be of interest for two reasons: (*a*) its long period of gestation, and (*b*) its frequent resistance to double ovariectomy during pregnancy. Stockard and Papanicolaou's observations (1917), however, made it clear that the uterine glands varied little during pseudo-pregnancy, though cyclic changes could be distinguished in the lining epithelium. Professor J. P. Hill has also pointed out to us that the uterine glands of the guinea-pig, unlike those of the rabbit and other mammals, take no part in placentation. Nevertheless, to check the condition of the sterile horn during pregnancy, four stages were obtained, at about 21 days, 39 days, 40 days and 45 days pregnant. The endometrium of the guinea-pig uterus (Plate 21, fig. 14) possesses a basal layer containing numerous glands, and above this, under the epithelium, a denser layer with comparatively few glands. In the sterile horn specimens, all very similar, the glands appeared less highly developed than in three unmated guinea-pigs examined at different phases of the cycle.

The Corpora Lutea.—In the absence of any marked reaction of the uterine glands which could serve as a diagnosis of luteal activity in the guinea-pig, an attempt was made to estimate the duration of corpus luteum activity in pregnancy from the histological appearance. Corpora lutea were examined from 22 pregnant guinea-pigs; 7 of these were classed as less than 4 weeks pregnant, while 15 others ranged from 36 days pregnant to full term. In the latter group the stage of pregnancy could be determined with a considerable degree of accuracy by X-ray examination of the epiphyses of the embryos.* Corpora lutea of ovulation were studied in 5 undated animals and in a guinea-pig 6 days post-partum.

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* Guinea-pigs have been aged from gross measurements and radiographic records of the state of ossification (as yet unpublished) by Dr. H. A. Harris. See a preliminary note on the relation of skeletal ossification in the hind limb to the index of cerebral value of Anthony and Cowper, 'J. Anat.,' vol. 63, pp. 267–276 (1929).

Examination and measurement of the material failed to reveal a clear sequence of histological changes in the corpora lutea of pregnancy; in comparison with those of other rodents they proved rather more difficult both to interpret and to measure, owing to their variability and less regular shape. Unlike the corpora lutea of mice, rats and rabbits, those of the guinea-pig hardly project from the surface of the ovary; they are imbedded in ovarian stroma and interstitial cells, and their shape, and to some extent possibly their size, is conditioned by the adjacent tissue.

(*a*) *The Corpora Lutea of Ovulation.*—The corpora lutea developing in the absence of copulation in the guinea-pig have been shown to affect the uterus physiologically, since in their presence uterine placentomata may be produced (Loeb, 1908), although, as mentioned above, the morphological reaction of the uterine glands appears to be negligible. It is possible, therefore, to correlate histological appearance with functional activity.

All but one of the specimens examined seemed recent and unshrunken and their diameters in section varied from 1·4 to 1·6 mm. Including the corpus luteum from GP 9 killed six days post-partum, which may be assumed to be functionally active, all this group of corpora lutea contained an irregular granulation of osmicated fat, coarser in GP 9 than in most of the others.

(*b*) *The Corpora Lutea of Pregnancy.*—Measurement of the corpora lutea indicated that the majority, having once reached their maximum size, underwent hardly any shrinkage until just before parturition. The amount of fatty cell inclusions varied, but in Flemming fixed material was not appreciably higher at the end of pregnancy than in its early stages (Plate 20, figs. 5 and 6). In the series examined the pregnancy corpora lutea showed most fat at 6 days post-partum, and it seems probable that the maximum fat period comes just after the end of pregnancy as in the mouse.

No accurately dated material was obtained for the first fortnight of pregnancy; the luteal function may well reach a maximum during this time and then decline, but histological examination makes it almost certain that there is no striking change such as occurs in the rabbit. The continuance of secretory activity in the cells of the later guinea-pig corpora lutea may be deduced from their changing fat and lipoid granulations. In many cases mitotic figures could be observed among the lutein cells, as Loeb (1911) has previously recorded, but these appear to be associated with cell degeneration rather than with cell division. Many corpora lutea, including ones from early pregnancies, contained an abundance of polymorphs and leucocytes, the significance of which it is difficult to estimate. Goormaghtigh (1927) believes that the secretion passes out through the lymphatic channels.

Typical specimens, including corpora lutea from the 4 guinea-pigs with one horn of the uterus sterile, are described below :—

Group I.—Under 4 weeks pregnant : 7 guinea-pigs ; average diameter of the measured corpora lutea, 1·83 mm.

SPG 10. Early pregnancy ; ovariectomised ; aborted the next day. Mean diameter in section, 1·66 mm.

The cells in Bouin fixed material are eosinophil and rarely vacuolated ; they show regular contours and arrangement. The lutein cell diameter is about 15 μ and that of their nuclei 7 μ. The blood vessels are dilated. Mitoses are rare or absent. Leucocytes are not common. After Flemming fixation the peripheral cells contain black fatty granules, but in the interior of the corpus luteum the cell granulation is brown.

SPG 14. Embryos estimated at 3 weeks old. Mean diameter, 1·88 mm. In comparison with SPG 10 the lutein cells appear less regular and somewhat smaller, though the difference in size is not marked. The nuclear diameter is about 6 μ and the cell diameter about 12 μ. The blood vessels are still dilated. A number of mitotic figures can be seen. The osmicated fat is still almost confined to the outside of the corpus luteum ; it is rather lighter than in SPG 10, but occupies a larger part of the section.

UPG 3. Early pregnancy, probably about 3 weeks ; one horn of the uterus sterile. Mean diameter, 2·05 mm.

The osmicated fat is coarser than in SPG 10 and 14 and present in irregular small masses and granules throughout the corpus luteum, but heaviest at the periphery. The cells contain abundant lipoids ; some cells are vacuolated, others contain mitotic figures. This corpus luteum from the appearance of its cells is more like SPG 14 than SPG 10, which was probably from a younger pregnancy.

Group II.—8 guinea-pigs 36 to 50 days pregnant ; average diameter of measured corpora lutea, 1·86 mm.

UPG 2. 39 days pregnant ; one horn of the uterus sterile. Mean diameter, 1·92 mm.

This corpus luteum is not strikingly different from that of UPG 2, but osmicated fat is rather more abundant and more evenly distributed throughout the corpus luteum. Leucocytes and cells belonging to the vascular connective tissue reticulum appear very abundant. Some of the lutein cells are vacuolated. Lipoids are similar to those of UPG 3.

SPG 24. 40 days pregnant; one horn of the uterus sterile. Mean diameter, 2·03 mm.

This is not very different from UPG 2, but the osmicated fat granulation is rather lighter and sparse in the interior of the corpus luteum. The vascularisation appears active and the lutein cells resemble those of SPG 10.

SPG 21. 41 days pregnant. Mean diameter, 1·45 mm.

The corpora lutea of these ovaries contrast with the others of the group in their smaller size and greater abundance of osmicated fats and lipoids. The latter are as heavy as in the post-partum corpus luteum GP 9, but the granulation is rather more regular. Both the cells and their nuclei appeared somewhat shrunken. All four corpora lutea were of the same type, and from their histological characteristics would be held to have reached the end of their functional period.

Group III.—6 guinea-pigs 45 to 58 days pregnant; average diameter of measured corpora lutea, 1·78 mm.

SPG 25. 45 days pregnant. Mean diameter, 1·72 mm.; one horn of the uterus sterile.

This corpus luteum resembles that of UPG 2, but the osmicated fat is coarser and more abundant (Plate 20, fig. 5).

SPG 16. 54 days pregnant. Mean diameter, 1·72 mm.

Here there is rather less osmicated fat than in SPG 25; it is irregular and mainly at the outside of the corpus luteum.

SPG 13. 58 days pregnant. Mean diameter, 1·80 mm.

This resembles SPG 16, but has coarser fat.

GP 10. Killed just before parturition. Mean diameter, 1·87 mm.

This corpus luteum did not appear shrunken, nor was osmicated fat heavy in comparison with earlier stages (Plate 20, fig. 6). It resembled that of SPG 25, 45 days pregnant. GP 8, killed at about the same time, showed rather smaller and more fatty corpora lutea, diameter 1·52 mm. Corpora lutea of the same size were found in SPG 3, which gave birth to two young the day after ovariectomy and suckled them for 6 weeks. In SPG 23, killed at 63 days pregnant, the corpus luteum diameter was 1·82 mm.

At 6 days post-partum the corpora lutea of pregnancy showed more osmicated fat than at any time previously; the diameter was 1·3 mm., the cells were shrunken, and the osmicated fat was in large globules and coarse granules.

In the present series therefore, only one guinea-pig out of 18 killed up to the 58th day of pregnancy shows definite signs of corpus luteum regression, and that at 41 days pregnant. Loeb (1911) refers to variations among pregnancy corpora lutea in this respect, even among those in the same ovary, but he nowhere defines his criteria of degeneration. From ovariectomy experiments it may be deduced that, in some cases at least, corpora lutea are not actively functioning (or that their secretion is no longer necessary for the maintenance of gestation), but, even in the guinea-pig, ovariectomy often terminates pregnancy (Herrick, 1928), though the effect is not always immediate.

VII. *Summary.*

(1) This paper deals with the changes in a sterilised horn of the uterus during pregnancy, and their relation to changes in the corpus luteum.

(2) In confirmation of Courrier and Kehl (1930, *a*), Knaus (1930) and Hammond, it was found that the sterile horn of the rabbit undergoes characteristic pregestational changes lasting 12 to 14 days and afterwards regresses. The glands and epithelium disintegrate and blocks of débris are found in the lumen. From 14 days pregnant till parturition, the corpora lutea in all but one of the rabbits examined showed a disappearance of fats, blackening with osmic acid from their inner region. The fat-containing zone decreased in size as pregnancy advanced and was reduced to a narrow rim at parturition. Since osmicated fats are found throughout sections of corpora lutea during the early days of pregnancy, their gradual disappearance and restriction in later stages probably indicates a decline in the elaboration of the luteal secretion. This may be correlated with the disintegration of the endometrium of the sterile horn.

(3) In the mouse, as in the rabbit, the changes in the sterile horn during the first half of pregnancy resemble those during pseudo-pregnancy, but during the second half, not regression, but an entirely new phase of development sets in. The uterine glands become greatly distended and their diameter may reach 250 μ to 300 μ. Regression, accompanied by some hæmorrhage in the stromal tissue, begins just before parturition. This second phase of glandular development may be correlated with the enlargement of the corpus luteum at about mid-pregnancy (Deanesly, 1930). The possibility that the endometrium of the sterile horn is influenced by fœtal or placental activity cannot be definitely excluded.

(4) No trace of such uterine change was found in the sterile horn of the pregnant rat.

(5) In the guinea-pig the morphological changes in the sterile horn during pregnancy are as slight as during pseudo-pregnancy, and consequently give no clue to the duration of luteal influence. Histological study of the corpora lutea after Flemming fixation suggests that the majority of them remain

functional throughout pregnancy; osmicated fat increases up to and after parturition.

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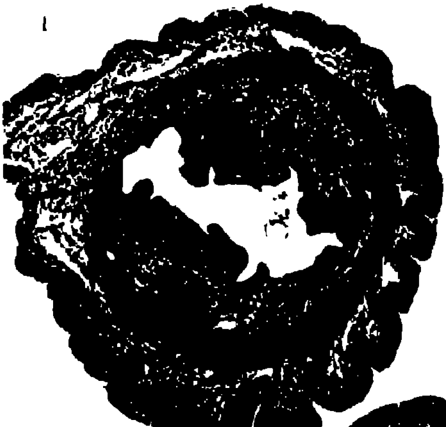

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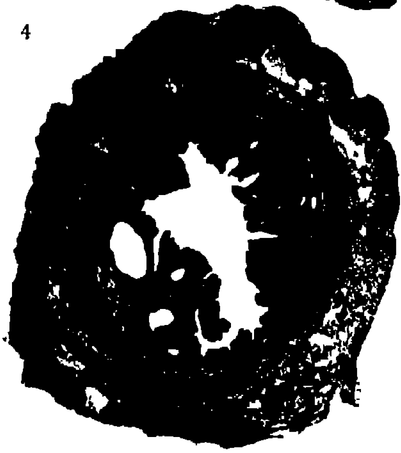

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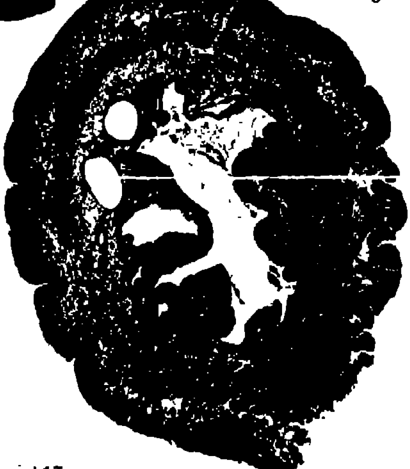

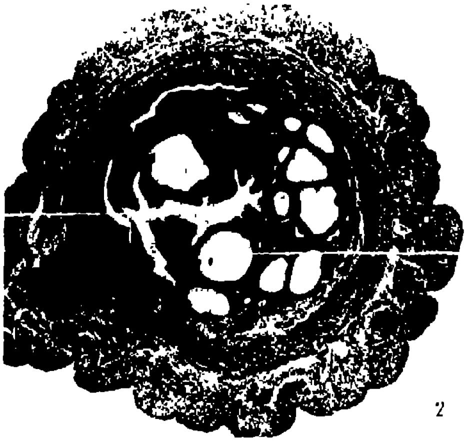

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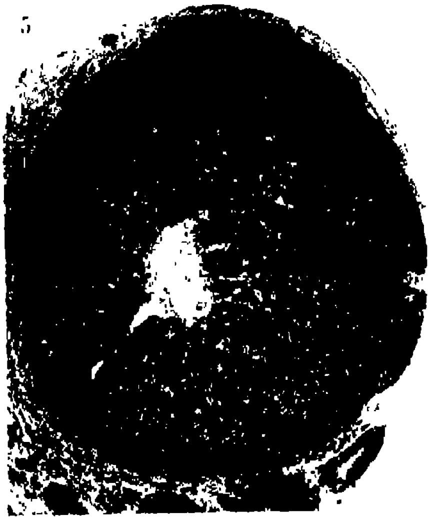

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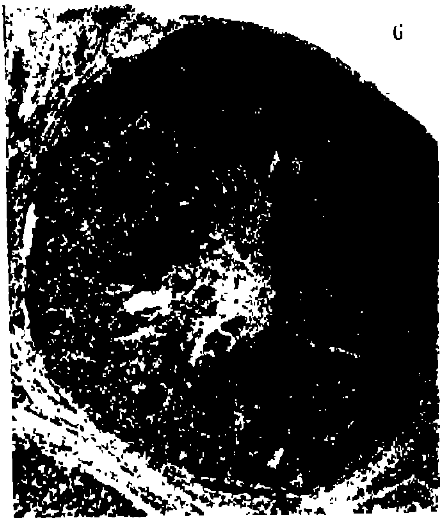

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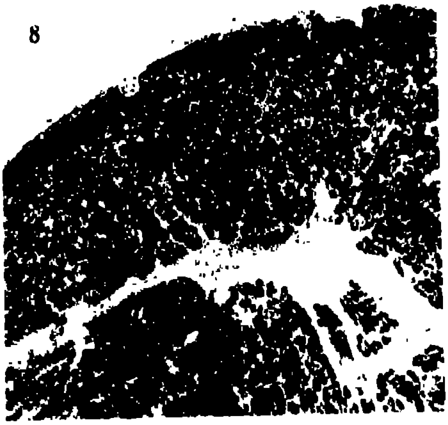

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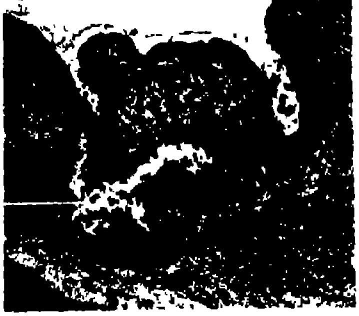

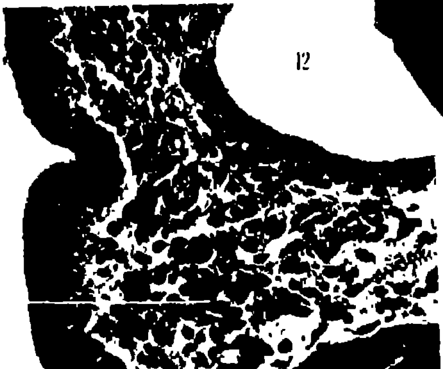
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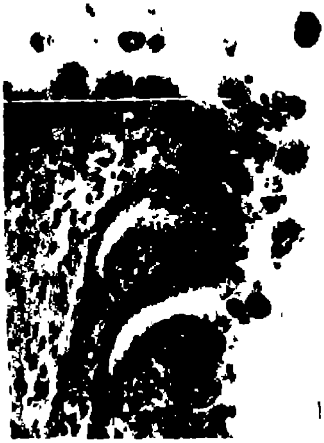

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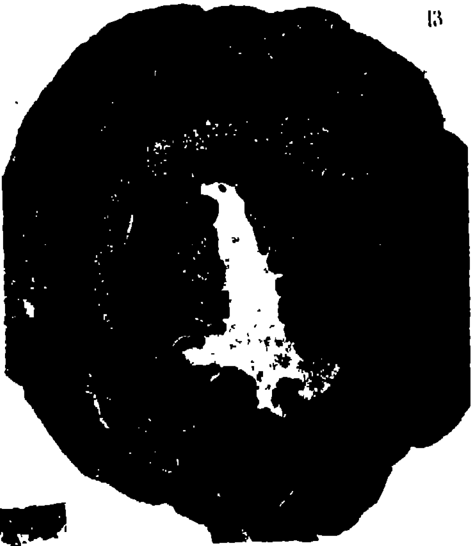
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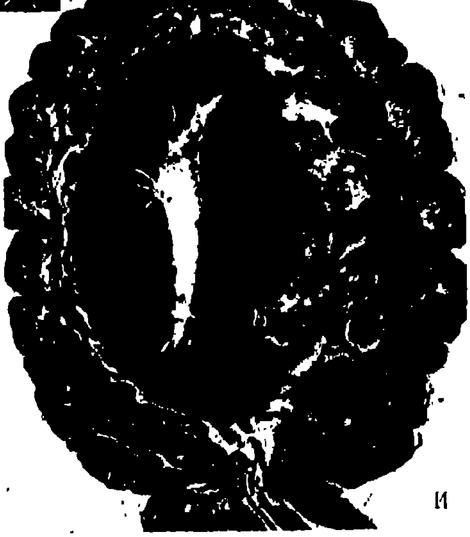
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DESCRIPTION OF PLATES.

Abbreviations.—D.G., distended gland; D., débris in lumen; G.C., giant cells formed from epithelial débris; R.B.C., red blood corpuscles; L, lumen of uterus.

PLATE 19.

FIG. 1.—Uterus of mouse, 11 days after sterile copulation, showing endometrial development reached during pseudo-pregnancy. × 45.

FIG. 2.—Sterile horn of mouse (UCP 2), at 17 days pregnant, showing the enormous distention of glands characteristic of the second phase of development in the sterile horn. × 28.

FIG. 3.—Sterile horn of mouse (SM 47) at 18 days pregnant. × 21.

FIG. 4.—Sterile horn of mouse (UCP 4) at 2 days post-partum. Uterus returning to normal. × 28.

PLATE 20.

7 μ sections through corpora lutea, fixed in Flemming's fluid and mounted unstained. × 25.

FIG. 5.—Guinea-pig 45 days pregnant (SPG 25).

FIG. 6.—Guinea-pig just before parturition (GP 10).

FIG. 7.—Rabbit 14 days pregnant (SM 9a). Disappearance of osmicated fat from inner region of corpus luteum.

FIG. 8.—Rabbit 23 days pregnant (UPR 5). Heavy osmicated fat throughout corpus luteum.

FIG. 9.—Rabbit 26 days pregnant (UPR 2). Osmicated fat reduced to a narrow rim.

PLATE 21.

FIG. 10.—26-day pregnant rabbit (UPR 2). Segment of endometrium of sterile horn showing disintegration of the glands and epithelium and accumulation of the débris in the lumen. × 21.

FIG. 11.—23-day pregnant rabbit (UPR 5). High-power view of fold of endometrium showing giant cells formed from the débris of the glands and epithelium. × 156.

FIG. 12.—18-day pregnant mouse (SM 47), showing presence of red blood corpuscles in the stromal tissue of the sterile horn. × 320.

FIG. 13.—19-day pregnant rat. Sterile horn showing complete absence of the glandular development found in the mouse. × 28.

FIG. 14.—45-day pregnant guinea-pig (SPG 25) Sterile horn showing quiescent endometrium. × 14.

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The Bronchial Vascular System in the Dog.

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[Plates 22–29.]

I. *Introduction.*

The distribution of the bronchial arteries (the *vasa nutritia* of Reisseissen) within the lung has been investigated by many anatomists but, beyond our knowledge that they are nutritional vessels to the bronchial tree (Reisseissen and Sömmering, 1808; Reisseissen, 1808, 1822; Luschka, 1863; Königstein, 1903), little is known of their significance for the maintenance of the normal functions of the lungs. At the moment their chief interest lies in the number of reports describing the changes in the bronchial blood vessels resulting from interference with the pulmonary vascular system and from lesions in the lungs.

Virchow (1847, 1851, 1856) produced evidence to show that long-standing obstruction of a pulmonary artery branch to one lobe led to an increased development of the bronchial arteries, although Karsner and Ash (1912) failed to obtain the increased development following pulmonary obstruction in a smaller branch. Christeller (1916) reviewed a number of cases showing increased bronchial artery development accompanying pulmonary stenosis and atresia. More recently Forssman (1920) and Schmidt and Barth (1924) have drawn attention to the sensitivity of the bronchial vascular endothelium in anaphylactic shock, and of considerable interest is the observation of Miller (1925) that the transitional zone from the bronchial to the pulmonary circulation in the bronchial tree is a favourite place for tubercles to develop. In this connection, Guillot (1845) discusses the formation of new blood vessels of the systemic circulation in the region of tubercular lesions.

Two years ago Professor E. B. Verney and one of us (I. de B. D.) were conducting experiments in this laboratory which involved the separation of the pulmonary from the systemic circulation in the dog, the two systems being perfused by separate pumps. In one experiment it appeared that blood was being transferred from the aorta to the left auricle through the bronchial vascular system. It became desirable, therefore, to inquire into the conditions which govern the bronchial blood supply. At an early stage in this inquiry it was evident that, owing to the variations in the origin of the bronchial arteries, a study of the gross and minute anatomy of the bronchial vascular system in each perfusion experiment would be necessary if anything approaching a correct interpretation of our results was to be expected. As a preliminary investigation to our perfusion experiments, we have examined the bronchial vascular system in a number of animals and the results form the subject of this paper. In it are also incorporated the anatomical findings on the perfused lungs used for physiological investigations. We have concentrated our efforts on two main issues: the first, the arrangement of the bronchial arteries and veins with a view to setting up apparatus for perfusion of the bronchial arteries and the collection of the blood from the bronchial veins; the second, an enquiry into possible arterial and venous communications between the bronchial and pulmonary vascular systems. It is clear that if arterial anastomotic channels exist between the two systems it will not be possible to separate completely the arterial blood of each.

Ruysch (1721), Haller (1756), Reisseissen (1822), Hoyer (1877), Küttner (1878), and Zuckerkandl (1883) were among the earlier workers on human lungs to describe an *arterial* anastomosis between the pulmonary and bronchial vascular systems, although Guillot (1845), Rossignol (1846), Adriani (1847), and more recently Miller (1900, 1906, 1925) found the two arterial systems to be independent. It is suggested by Miller (1925) that the *vasa vasorum* from the bronchial arteries to the branches of the pulmonary artery may be mistaken for anastomotic branches. Zuckerkandl called the twigs of the pulmonary artery which go direct to the air tubes and there anastomose with the bronchial artery—*rami bronchiales arteriæ pulmonalis.*

Ample evidence of a *capillary* anastomosis between the two systems has been produced by Reisseissen (1822), Kolliker (1852), Hyrtl (1873), Küttner (1878) and Miller (1906, 1911, 1925, 1925, *a*). Cohnheim and Litten (1875) at one time denied the presence of a free exchange of blood between the two systems, but at a later date Litten (1879) reinvestigated the problem and retracted from this position, Cohnheim (1882) accepting his former co-worker's opinion. Küttner (1878) injected the bronchial vessels from the pulmonary artery and Zuckerkandl (1881) succeeded in injecting the respiratory capillary network from the pulmonary artery, the pulmonary veins or the bronchial arteries. Miller (1906) demonstrated that the bronchial blood vessels are directly connected with the pulmonary veins and can be only partially injected from the pulmonary artery when the pulmonary vein is clamped and then by a backward flow along the venous radicles which arise from the bronchi; again, Rossignol (1846), Adriani (1847), Miller (1906), Ghoreyeb and Karsner (1913), and others have shown that injection of a dye into the aorta leads to its appearance in the pulmonary veins and superior azygos vein.

The *capillary* anastomosis does not take place in all parts of the lung. According to Miller (1925) the bronchial arteries supply the bronchial tree as far as the small bronchioles, but with the appearance of alveoli along the walls of the bronchioles the bronchial arteries as a distinct set of vessels disappear, the terminal network of capillaries formed by them uniting with that of the pulmonary artery in the region of the *bronchioli respiratorii*; beyond this region the air tubes and air spaces are supplied by the pulmonary artery. (Le Fort (1858), Luschka (1863), Küttner (1878), Zuckerkandl (1881) state that part of the respiratory capillary network is supplied by branches from the bronchial arteries.) The blood from the bronchial artery supplying the hilum, with its adjacent pleura and the first two dividing points of the bronchial tree, drains into the azygos, hemi-azygos or one of the intercostal veins, whereas that supplying the remaining bronchi and bronchioli as far as the capillary network in the walls of the *bronchioli respiratorii* empties into small branches of the pulmonary vein. According to Küttner (1878) mixed blood supplies the alveoli in the region of the bronchiolus respiratorius and ductulus alveolaris; this point is also discussed by Hyrtl (1873).

Sappey (1889) believed the bronchial artery extended to the termination of the lobules and it is interesting to note that Miller (1911) found that the last remnant of blood which is brought to the bronchus by the bronchial artery drains into the venous radicles which take their origin from the distal end of the ductulus alveolaris.

Regarding the *venous* communications between the larger branches of the pulmonary and the bronchial veins, Reisseissen and Sömmering (1808), Le Fort (1858) and Zuckerkandl (1881) found them to be present, but Luschka (1863), Hyrtl (1873) and Sappey (1889) denied their existence Hyrtl (1873) considered the so-called bronchial veins, which have been described as emptying into the pulmonary veins, to be small veins of the latter type, but that true bronchial veins drain the bronchial glands and the larger bronchi. According to Melnikoff (1923) the true bronchial veins are not well developed.

The more recent work tends to show that the arterial connections between the pulmonary and bronchial systems are probably non-existent, but there is undoubtedly a capillary anastomosis. It must be remembered, however, that most of the earlier investigators worked on human lungs, a few on dogs' lungs, and Miller (1906, *a*, 1908) has demonstrated a wide difference between the distribution of the bronchial arteries in different species; this especially applies to the blood supply of the *pleura pulmonalis* (see also Backman, 1923).

II. *Methods.*

For the purpose of the investigation we have injected the pulmonary artery, the aorta, one bronchial artery, the pulmonary veins or the azygos vein at level D10 with a gelatin-dye or a barium sulphate-gelatin mixture subsequent to a perfusion with saline at 40° C. Following these procedures, the larger blood vessels of the lungs have been examined in the first instance *in situ* with or without the aid of a hand lens, and finally the lungs have been sectioned for microscopic examination. The lungs injected with the barium sulphate-gelatin mixture, after being radiographed, have also been sectioned and the distribution of the injection mass observed under the microscope. Such a complete examination has been made in 26 animals, a partial examination in 15 others. After trials, the most satisfactory injection masses have been found in the Prussian blue-gelatin and the carmine-gelatin prepared according to the directions given by Gross (1921). Injections into the aorta or bronchial arteries are carried out with a modified Huber (1906) apparatus at pressures from 150 to 400 mm. Hg, and into the pulmonary artery, pulmonary veins, right auricle or superior azygos vein from 40 to 200 mm. Hg. The heart is clamped at the auriculo-ventricular groove to prevent passage of the injection mass from one system to the other through the cardiac chambers. The time taken for the injection varied from 3 to 15 minutes, the temperature being approximately 40° C. The tissues injected with the dye solution are hardened—first in equal parts of rectified spirit and water with the addition of 1 per cent. hydrochloric acid, then in rectified spirit and, lastly, in absolute alcohol.

The specimens for radiographic study are injected with a 40 per cent. $BaSO_4$ and 12·5 per cent. gelatin mixture into the aorta at 150 to 400 mm. Hg to fill the bronchial arterial system, or into the right auricle or azygos vein to fill the bronchial veins. The radiographs of the injected vessels are taken on Kodak X-ray films in double wrappers. The specimens are placed on cotton-wool in a wooden box, fitted with a bottom of thin sheet aluminium. The film is placed on the floor and the box containing the specimen laid upon it, the X-ray tube being fixed 5 feet above the specimen so as to reduce distortion. The close contact of the specimens with the film without intensifying screens avoids the grain of the latter and gives sharp detail of the finest vessels. The average exposure has been 5 seconds, using 50 milliamperes of current at a tension of 60 kilovolts peak. The tissues injected with 40 per cent. barium sulphate gelatin mixture were fixed in 10 per cent. formol solution, the p_H value of which was adjusted to 6·0 with sodium acetate using methyl-red indicator. This technique was adapted from Jacquiert (1930).

III. *The Bronchial Arteries and Veins.*

A gelatin-dye injection into the aorta or a backward injection of the bronchial arteries from the left auricle rendered a dissection of these vessels a comparatively easy matter. (A back injection of the bronchial arteries from the left auricle has been used by Rossignol (1846) and Adriani (1847).)

We have found that the bronchial arteries arise in general in one of two ways :—

(*a*) The *posterior bronchial artery* arises from the sixth intercostal artery and divides into a right and left branch, twigs from each supplying the œsophagus. The *anterior bronchial arteries* arise from the internal mammary and from the pericardial arteries of the same side which sink with the mediastinal and tracheo-œsophageal branches into the lung hilus. This is nearly identical with the arrangement described by Küttner (1878).

(*b*) A branch from the right fifth or sixth intercostal artery to the right lung and one or more twigs direct from the first part of the descending thoracic aorta down to D7 or a single twig from the left sixth intercostal artery to the left lung comprise the posterior bronchial arteries (Plate 22, fig. 1), which also supply a portion of the œsophagus. The anterior bronchial arteries arise from the internal mammary artery, the mediastinal and pericardial arteries of the same side.

Twigs from the pericardiaco-phrenic artery, from the aortic vessels in the *ligamentum latum pulmonale* and from the small artery supplying each vagus have been seen entering the hilus of the lung. In one animal a right posterior bronchial artery arose directly from the aorta in addition to the usual branch coming off from the sixth intercostal artery. The posterior bronchial arteries are far larger than the anterior and send relatively large branches to the bronchial glands, and in addition supply twigs to the pulmonary branches of the vagus. In a well-injected specimen communicating branches between the œsophageal, mediastinal, pericardial and bronchial arteries can be made out. The *ligamentum latum pulmonale* in the region of the lung roots receives twigs from the bronchial artery branches.

We find that small twigs arising from the bronchial, pericardial and mediastinal arteries sink into the lung hilus and spread over the visceral pleura in this region, they also run in the *ligamentum latum pulmonale* together with branches arising directly from the aorta (fig. 8). Küttner (1878) states that the bronchial and pulmonary arteries in the dog can be filled from the pleural mediastinal twigs, and Zuckerkandl (1883), Melnikoff (1923) and Backman (1923) discuss the presence of such a collateral circulation in man. The wide variations in the origin of the bronchial arteries encountered from one animal to another are also found in human lungs (Haller, 1756; Krause, 1868: Nakamura, 1924).

With the aid of dye-gelatin injections into the superior azygos vein it is found that the posterior bronchial veins on the right side enter the azygos vein as it curves forwards to enter the superior vena cava at the level of the fifth or sixth thoracic vertebra; a few twigs may enter the azygos vein lower down as far as the level of the eighth thoracic vertebra. On the left side the bronchial veins drain into the azygos vein, an intercostal vein or branches of the œsophageal veins which eventually empty into the azygos vein. The mediastinal, œsophageal and bronchial veins anastomose with one another. On one occasion some of the bronchial veins from both sides united to form a large trunk which entered the azygos vein at the level of the seventh thoracic vertebra. The anterior bronchial veins, which are small and difficult to trace, for the most part appear to drain into the internal mammary vein.

These observations indicated the lines upon which the experiments would have to be planned to ensure perfusion of the bronchial arteries and collection of the blood from the bronchial veins; further, they made us fully aware of the difficulties which would be encountered in separating completely the bronchial arterial and venous blood from that supplying and draining adjacent structures. At the moment it is sufficient to stress that the posterior bronchial arteries, which are the chief supply to the air tubes, arise from the descending thoracic aorta down to D7, whereas the posterior bronchial veins eventually drain into the superior azygos vein and azygos vein down to level D8.

We have been unable to trace bronchial venous twigs to the right auricle, although a few sometimes enter the superior vena cava in its course between the right auricle and entrance of the superior azygos vein.

It will be seen that perfusion of the part of the thoracic aorta lying between the level D8 and a ligature tied just distal to the left subclavian artery, will supply blood to the bronchial tree fed by the posterior bronchial arteries which is the major supply to this area; and a cannula in the superior azygos vein, the azygos vein being ligatured just below the level of the eighth thoracic vertebra, will collect most of the blood derived from the bronchial veins which emerge from the lung hilus. It should be stated, however, that unless certain precautions are taken, systemic vascular territories other than the bronchial will be involved in such a perfusion; they will be described later.

IV. *Arterial, Venous and Capillary Communications between the Pulmonary and Bronchial Vascular Systems.*

We have repeatedly attempted to demonstrate arterial communications between the pulmonary and bronchial vascular systems without success. If such wide anastomotic channels, from 0·135 mm. to 0·42 mm. in diameter, between the bronchial and pulmonary arterial branches exist in the dog, as Hoyer (1877) states to be the case in man, it would be expected that a gelatin-dye mixture would pass easily into the pulmonary artery when the bronchial arteries are injected; this, however, does not take place. Additional evidence of the absence of such an anastomosis is afforded by the radiographs which, taken after aortic injection with the barium sulphate-gelatin mixture, reveal clearly the bronchial arteries which follow the main air tubes, but there is no passage of the material into the adjacent lung tissue; the smallest vessels which show up on the radiographs have a diameter of 70 μ, and their spiral course first mentioned by Haller (1754) is characteristic. Microscopic examination of the lungs shows that the barium sulphate-gelatin reaches vessels which are distended to 17 μ in diameter, yet the pulmonary arteries are uninjected. This suggests that our injection material reaches the arterial vessels which immediately precede the capillaries, for Miller (1925) shows in his beautiful picture of the capillary network within the walls of a bronchiolus, a small bronchial artery of 25 μ in diameter quickly reducing to one of approximately 10 μ as it gives off branches which merge into capillaries. Taking all the evidence into account, we are forced to the conclusion that there is no extensive arterial communication between the bronchial and pulmonary vascular systems in the dog, although our results do not exclude the presence of an anastomosis of the smallest arteriole twigs of the two systems.

With regard to the *capillaries*, the injection material has been in general too thick to be able to enter these vessels at the pressures used, but on a few occasions we observed capillaries in the region of a bronchus which were filled with Prussian blue-gelatin injected from the aorta. We have, however, repeatedly observed saline flowing from the superior azygos vein and left auricle during the preliminary irrigation from the aorta, and in some cases the gelatin from the barium sulphate-gelatin mixture has separated out with a small amount of the barium salt and filled some of the capillaries, but is then not in sufficient concentration to give a detectable shadow on the radiograph.

It has also been impossible to demonstrate a definite anastomosis between the *veins* of the two systems, as described by Zuckerkandl (*loc. cit.*) for human lungs. Injection of the azygos vein with barium sulphate-gelatin only leads to filling of a limited field of vessels situated round the larger bronchi, and the pulmonary veins have been free from the main mass of injection material, although in some cases (not all) the gelatin with a small quantity of barium sulphate has reached them. The smallest bronchial veins in these cases have been distended to 70 μ in diameter.

We interpret these results to mean that extensive venous anastomotic channels greater than 70 μ in diameter between the two systems are not present. It is not possible to be definite with regard to the smaller veins; in this connection it has been shown that the small pulmonary venules into which the capillaries of the network within the walls of a bronchiolus drain, vary from 10 to 40 μ in diameter; these figures are derived by measurement of the vessels shown in fig. 3 of Miller's paper (1925).

V. *Significance of Anatomical Findings in relation to Physiological Experiments.*

Since much of our information of the distribution of the bronchial vascular system has been obtained from preparations previously used for perfusion experiments, it is proposed to present the radiographs of these preparations so that they may be compared with the physiological findings which will be described later. This procedure is necessitated by the fact that we used three different methods, in which the extent of the systemic vascular system perfused, including the bronchial, differed to a marked extent; moreover, the physiological results showed features peculiar to each.

The first group of experiments involved the perfusion of the pulmonary circulation with one pump and the thoracic aorta between levels D4 and D8 with a second pump. The animal is lightly anæsthetised with a mixture of chloroform and ether in equal parts, it is then bled to death from a carotid artery and the thorax opened by splitting the sternum in the mid-line. The arch of the aorta is tied with a strong ligature just lateral to the point where the left vagus passes over the aortic arch, and the brachiocephalic artery and the left subclavian artery are also ligatured. The lower half of the animal is then removed at level of D12 and the spinal canal plugged with cotton-wool soaked in hot paraffin wax, of m.p. 55° C. The muscles of the neck are surrounded with a strong chain, which is tightened by a specially devised mechanism in order to prevent subsequent bleeding from these structures; the head of the animal is then removed by section just above this clamp, following which the skin and most of the dorsal musculature are removed. The œsophagus is tied with a strong ligature at the level of the origin of the left subclavian artery, and also at levels of D5 to D8. Cannulæ are placed in the pulmonary artery, pointing peripherally, in the superior azygos vein, in the left auricle and in the descending portion of the thoracic aorta at level of D8, pointing headwards. The azygos vein at level D8 is tied and the auricular ventricular groove clamped.

In a few experiments the cannula has been placed in the right auricle instead of in the superior azygos vein, but in these cases the superior vena cava is ligatured above the entry of the superior azygos vein. The pulmonary vascular system is washed through with defibrinated blood, the blood being collected from the left auricle; the systemic circulation, which includes the bronchial vascular system, the intercostal muscles and part of the longus colli muscle, is similarly treated by perfusing the aorta with defibrinated blood at approximately 100 mm. Hg, the washings being collected from the superior azygos vein or right auricle as the case may be. This may be designated as a lung-thorax-œsophagus preparation (L.T.O.). The extent of the perfusion of the systemic system is seen in the radiographs (figs. 2 and 2A, Plates 23, 24), details of which are given in the legends to the figures.

Although most of the dorsal musculature is removed before perfusion, quite a large vascular bed is still supplied by the dorsal branches of the intercostal arteries. We felt reasonably certain that blood did not supply the territory very far distal to the intercostal artery ligatures near the vertebral column, since no oozing of blood occurred at the cut end of the arteries in the mid-sternal line, or in some experiments just distal to the ligatures if the thoracic parietes were removed. Injection with barium sulphate-gelatin at high pressures does not bear out this supposition, for the intercostal arteries in some preparations receive the injection mass. We believe that in these cases the injection material may have forced its way past the ligatures and reached portions of the vascular system which had not been perfused; we are, however, unable to make a definite statement on this point; such uncertainty is obviously a drawback to the injection method for assessing the extent of the vascular bed perfused, but at the moment there does not seem to be a better alternative. This disadvantage is lessened by performing a large number of experiments and assessing the results as a whole, but it is desirable to mention that the injection mass might well reach vascular territories which were not actually perfused. In the L.T.O. group we varied the technique slightly and in some experiments endeavoured to remove most of the thoracic parietes (figs. 3 and 3A, Plates 25, 26), leaving only five intercostal spaces, the aorta and that portion of œsophagus which remained attached to the corresponding vertebræ. In this way we hoped to make the bronchial vascular system a large proportion of the whole perfused systemic circulation.

In the second group of experiments, the intercostal arteries and veins are ligatured, the aorta, œsophagus and lungs being removed from the thorax by careful dissection of the posterior mediastinal contents from the bodies of the vertebræ. At this stage great care is taken to prevent damage to the right bronchial artery, which comes off from the fifth or sixth right intercostal artery, and to the intercostal arteries central to the placed ligatures. The pulmonary vascular system is washed through with defibrinated blood as before. The systemic circulation, now consisting of the blood vessels supplying a portion of the œsophagus, the posterior mediastinum and the bronchial tree, is also washed through, and the dorsal branches of the intercostal arteries, which have of necessity been cut, are tied with any other bleeding points. The length of the œsophagus receiving blood from the aorta varied from 2·5 to 8·0 cm. As practice was gained in the operation, shorter lengths of the œsophagus were left for perfusion; we term these, lung-œsophagus (L.O.) preparations (fig. 4, Plate 27).

An examination of these radiographs shows there is still a considerable vascular bed other than the bronchial involved in the perfusion, because of the rich blood supply to the œsophagus from the aorta direct, and from the bronchial arteries. We proceeded, therefore, to a third group of experiments in which cannulæ were inserted into one or more of the bronchial arteries. After a few trials it was found possible to tie off the small œsophageal arterial and venous twigs without damage to the bronchial arteries. These twigs in some instances are extremely short (fig. 6), but by drawing the œsophagus downwards and snipping its connective tissue sheath all the way round, ligatures could be placed on the vessels and tied with the sheath included. This operation is carried out at the level of the lung roots, working from below; on releasing the œsophagus it retracts upwards, leaving a clear operative field for insertion of the cannulæ into the bronchial arteries.

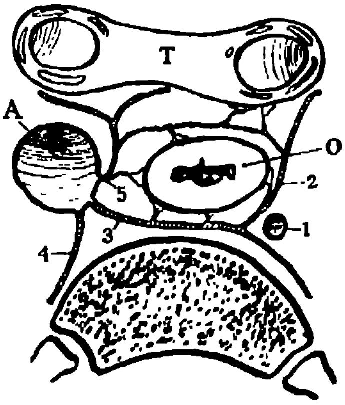

Fig. 6.—Bronchial arterial network surrounding the œsophagus. A = aorta; O = œsophagus; T = bifurcation of trachea; 1 = azygos vein; 2 = right posterior bronchial artery; 3 = right intercostal artery; 4 = left intercostal artery; 5 = left posterior bronchial artery.

In those animals which have only one left posterior bronchial artery a cannula is inserted into it after slitting the aorta and introducing the cannula through the arterial orifice; if two arteries are present on the left side they generally come off close together and a cannula is introduced into the portion of the aorta from which they arise, all other branches being tied. On the right side the cannula is inserted into the bronchial artery just after it leaves the intercostal artery (fig. 7). The œsophagus is drawn in the diagram in order to show its relation to the bronchial arteries, but in practice it is removed before

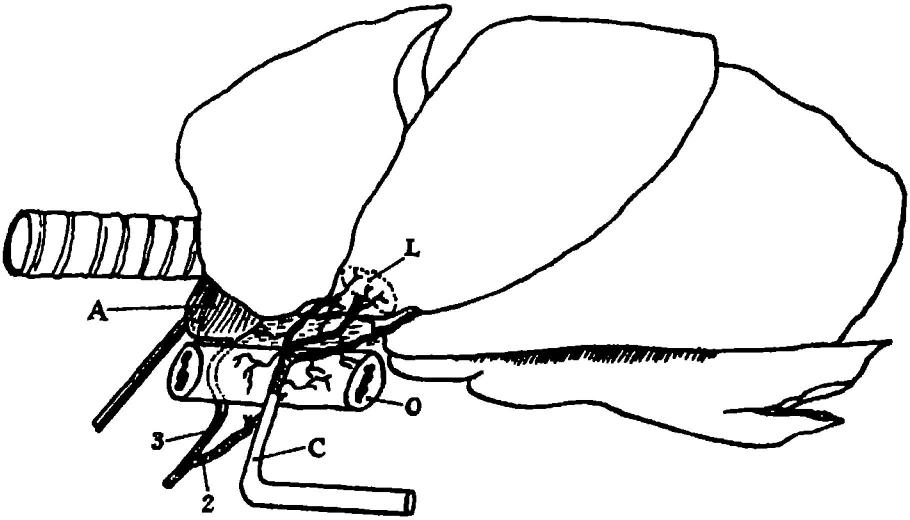

Fig. 7.—Diagram illustrating the position of the cannula inserted into the right posterior bronchial artery. C = cannula in right posterior bronchial artery; 2 = origin of posterior bronchial artery; 3 = right intercostal artery; A = portion of aorta pulled to right; O = œsophagus; L = lymphatic gland.

the cannula is inserted. The bronchial cannulæ are joined in parallel by rubber tubing and connected to the output side of the systemic pump. The radiograph (fig. 5, Plate 28) shows the result of injecting the right and two left posterior bronchial arteries with barium sulphate-gelatin, subsequent to a perfusion experiment.

VI. *Histological Findings.*

Under this heading we do not intend to describe in detail the results of the examination of some hundreds of sections obtained from the perfused and injected lungs, because, as far as our observations go, they confirm the full accounts given by Miller (*q.v.*). We do, however, wish to present further evidence that the masses injected, subsequent to the perfusion experiments, fill the bronchial arteries supplying the bronchial walls, including the submucosa, the walls of the pulmonary artery, and the pulmonary nerves (figs. 9 to 13, Plate 29). The bronchial arteries in the photographs show up black or appear granular, depending upon the type of light filter used.

Reisseissen (1822) states that the bronchial arteries supply the pulmonary nerves, and the close connection between branches of the bronchial arteries and the pulmonary nerves in the sections examined is particularly striking. Frequently a nerve bundle is flanked by two arteries and in some cases a small arterial branch may be seen within the nerve sheath (figs. 11 and 12, Plate 29). We consider therefore that in perfusion experiments of the pulmonary circulation simultaneous perfusion of the bronchial vascular system serves to maintain the blood supply to the bronchial walls and pulmonary nerves. It might well be that in lungs perfused through the pulmonary artery alone, the pulmonary nerves receive an insufficient blood supply, and in consequence lose excitability and conductivity. If this were so it might explain the failure of some investigators to obtain evidence of pulmonary vasomotor nerves, using a perfused preparation.

Summary.

(1) The course and distribution of the bronchial arteries in the dog are described.

(2) Examination of the bronchial and pulmonary vascular systems reveals no evidence of extensive arterial or venous communications between the two systems, the capillaries and possibly the smallest arterioles and venules serving as the only means of direct communication.

(3) Three methods of perfusing the bronchial vascular system are described. The first necessitates the concomitant perfusion of the thorax and œsophagus; the second involves only a small portion of the œsophagus, and the third is an isolated bronchial vascular system perfusion. In all three methods the pulmonary vascular system is perfused separately.

(4) The pulmonary nerves gain their nutrition from the bronchial vascular system.

We are indebted to Miss E. Stewart for help with the radiographs, and to Mr. R. H. Baron for the preparation of the histological specimens.

The expenses of the research have been defrayed in part by a grant to one of us (I. de B. D.) from the Government Grant Committee of the Royal Society, to whom we express our thanks.

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DESCRIPTION OF PLATES.

Plate 22.

Fig. 1.—Dog, 10·0 kg. Prussian blue-gelatin injection into the aorta at 200 mm. Hg. for 10 minutes at 46° C. to demonstrate the bronchial arteries. The lungs have been removed. *Upper.*—Two left posterior bronchial arteries arise from the aorta and are distributed to the bronchial gland, the trachea, the œsophagus and the vagus. *Lower.*—The right posterior bronchial artery arises from the fifth right intercostal artery and supplies the same structures as the above on the right side. A = aorta, O = œsophagus. The bronchial arteries and their main branches have been inked over.

Plates 23 and 24.

Figs. 2, 2a.—Dog, 9·0 kg. Radiographs of lung-thorax-œsophagus perfusion preparation. The thoracic aorta was perfused with blood between levels D4 and D8 simultaneously with perfusion of the pulmonary artery. At the end of the perfusion experiment the aorta and pulmonary artery were perfused with warm saline, at 150 and 30 mm. Hg respectively, for 6 minutes. The aorta was then injected through the same cannula with 40 per cent. barium sulphate + 12·5 per cent. gelatin mixture, at 150–300 mm. Hg for 10 minutes at 40° C. After refrigeration to enable the injection mass to set, the lungs, aorta and œsophagus were separated from the thoracic parietes and each part radiographed by the method described in the text.

Fig. 2.—The vascular bed of the thoracic parietes. *Fig.* 2a.—the bronchial vascular system of the lungs, with the aorta (lower left) showing *vasa vasorum* and the œsophagus (lower right) showing injected vessels.

Plates 25 and 26.

Figs. 3, 3a.—Dog, 13·0 kg. Radiographs of lung-thorax-œsophagus perfusion preparation. Subsequent to the double perfusion experiment the aorta and pulmonary artery were perfused with warm saline, at 100–150 and 30 mm. Hg respectively, for 9 minutes. The aorta was then injected with barium sulphate-gelatin mixture, at 140–300 mm. Hg for 9 minutes at 40° C. The lungs and aorta + œsophagus were separated from the five intercostal spaces of the preparation.

Fig. 3.—Radiograph of thoracic parietes. *Fig.* 3a.—Radiograph of the lungs with the aortic and œsophageal segments below. The œsophageal arterial twigs from the aorta stand out clearly.

Plate 27.

Fig. 4.—Dog, 12·0 kg. Radiographs of lung-œsophagus perfusion preparation, œsophagus above, aorta and lungs below; the right *lobus cardiacus* has been removed. Subsequent to the double perfusion experiment the aorta was perfused with warm saline, at 100–200 mm. Hg for 4 minutes. The pulmonary artery was not perfused with saline. The aorta was then injected with barium sulphate-gelatin mixture, at 200–240 mm. Hg for 12 minutes. After refrigeration the œsophagus was separated from the lungs and aorta, and the aorta raised to show the origin of the bronchial arteries. The œsophageal segment is shown at the top of the radiograph.

PLATE 28.

FIG. 5.—Dog, 18·0 kg. Radiograph of isolated lung perfusion preparation. Cannulæ were inserted into the pulmonary artery, the right posterior bronchial artery and a segment of the aorta which gave origin to two left posterior bronchial arteries. Simultaneous perfusion of the pulmonary and bronchial arteries was then carried out. At the end of the experiment the bronchial arteries were injected with bismuth oxychloride 20 per cent. + gelatin 11 per cent., at 400 mm. Hg for 15 minutes at 45° C. The blood vessels were not washed through with saline. After refrigeration the aortic segment was removed and the preparation radiographed.

PLATE 29.

FIG. 8.—Radiograph of bronchial arterial vessels (injected with bismuth oxychloride-gelatin) lying in the *ligamentum latum pulmonale* between the right diaphragmatic and intermediate lobe. From the same preparation as shown in fig. 5.

FIG. 9.—Photomicrograph of portion of bronchial wall showing blood vessels passing through the muscle mesh. From the central portion of the left diaphragmatic lobe of the same preparation as shown in figs. 5 and 8. × 56.

FIG. 10.—Photomicrograph showing bronchial arterial branches entering the bronchial submucosa. From 20·0 kg. dog, right diaphragmatic lobe. Carmine 3·0 per cent. + gelatin 11·3 per cent. injected into the aorta at 90 mm. Hg for 10 minutes at 40° C. Prior to the injection the aorta was perfused with warm saline at 80 mm. Hg. Section unstained. × 56.

FIG. 11.—Photomicrograph of a nerve bundle with a bronchial arterial branch granular in appearance, situated at " v. o'clock " just within the nerve sheath. The nerve lies in the adventitia of a bronchial tube 2 mm. in diameter. From lung-œsophagus perfusion preparation, dog 12·5 kg., upper margin of left diaphragmatic lobe. Subsequent to perfusion with warm saline at 120 mm. Hg for 3 minutes, the thoracic aorta was injected with barium sulphate-gelatin at 200 mm. Hg for 10 minutes at 40° C. × 150.

FIG. 12.—Photomicrograph showing in the centre of the field a nerve flanked by two bronchial arteries. From same preparation as fig. 11. × 56.

FIG. 13.—Photomicrograph of bronchial arteries (black) in the walls of a bronchial tube. Lung-thorax-œsophagus perfusion preparation, dog 9·3 kg., proximal portion of left diaphragmatic lobe. Subsequent to perfusion of the aorta with warm saline the thoracic aorta was injected with warm barium sulphate-gelatin for 5 minutes at 400 mm. Hg. × 17.

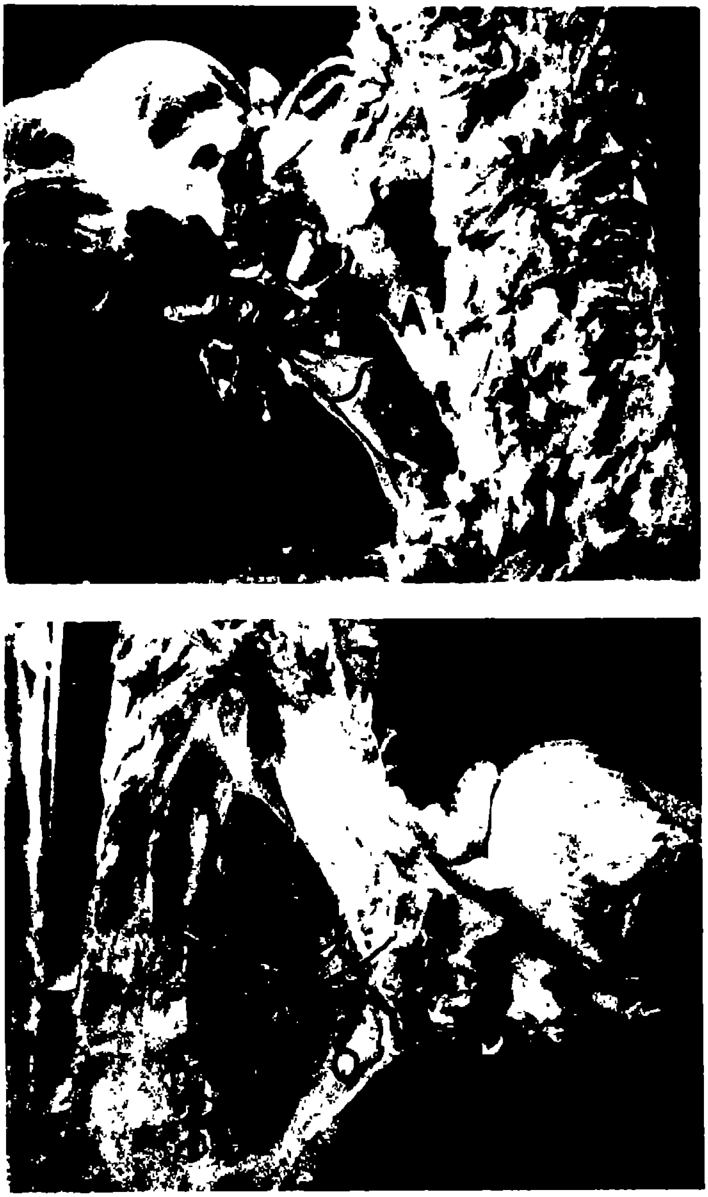


Fig. 1.

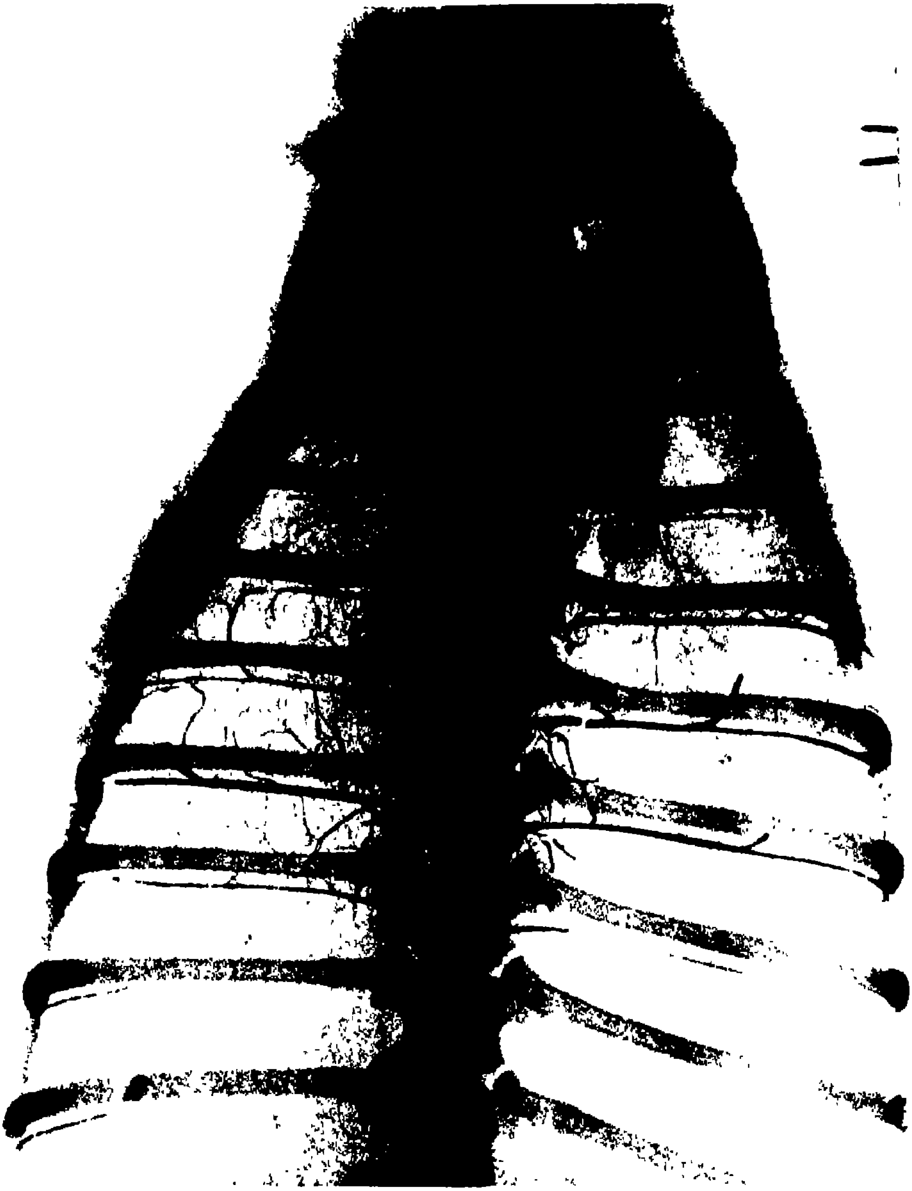
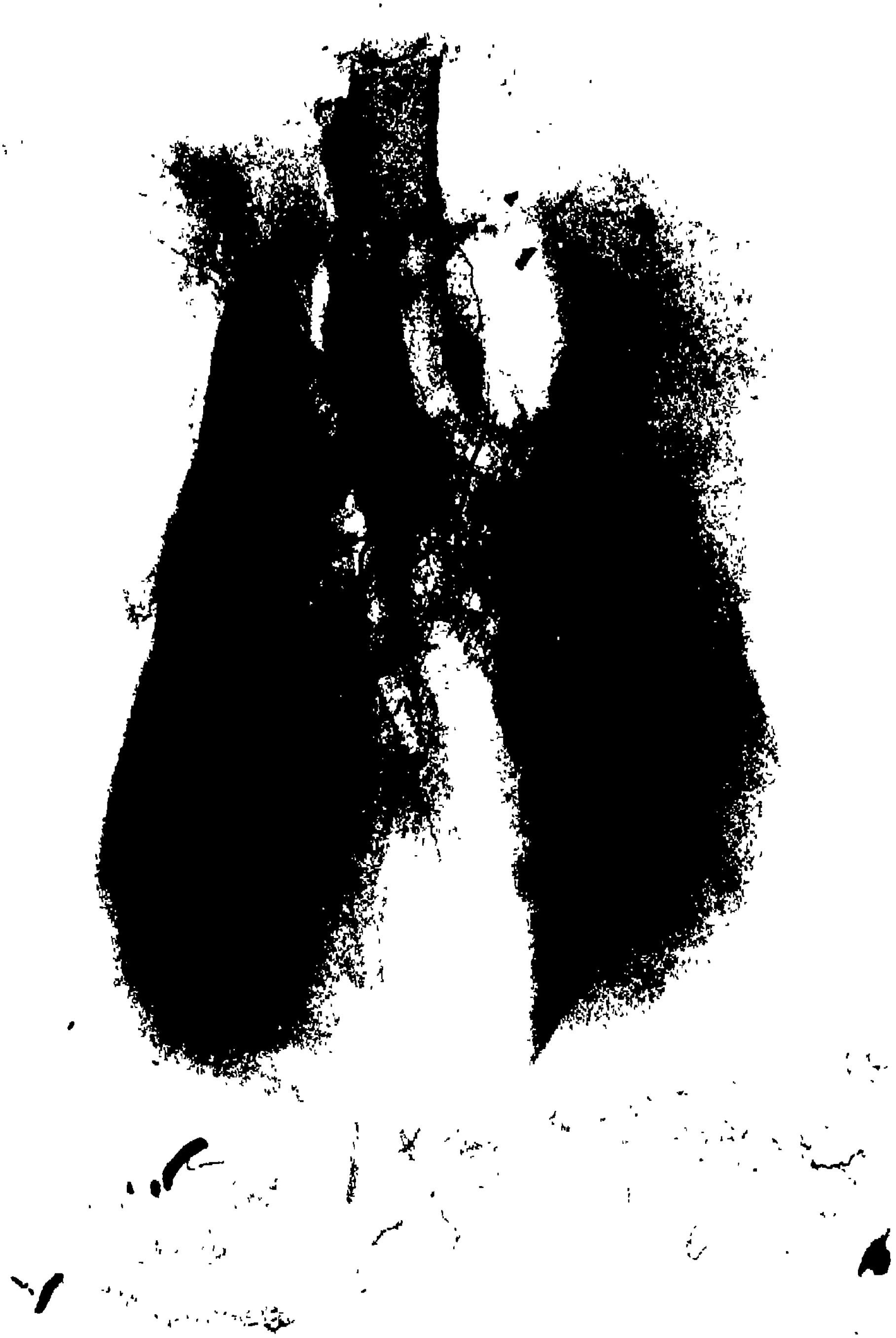


Fig. 2.



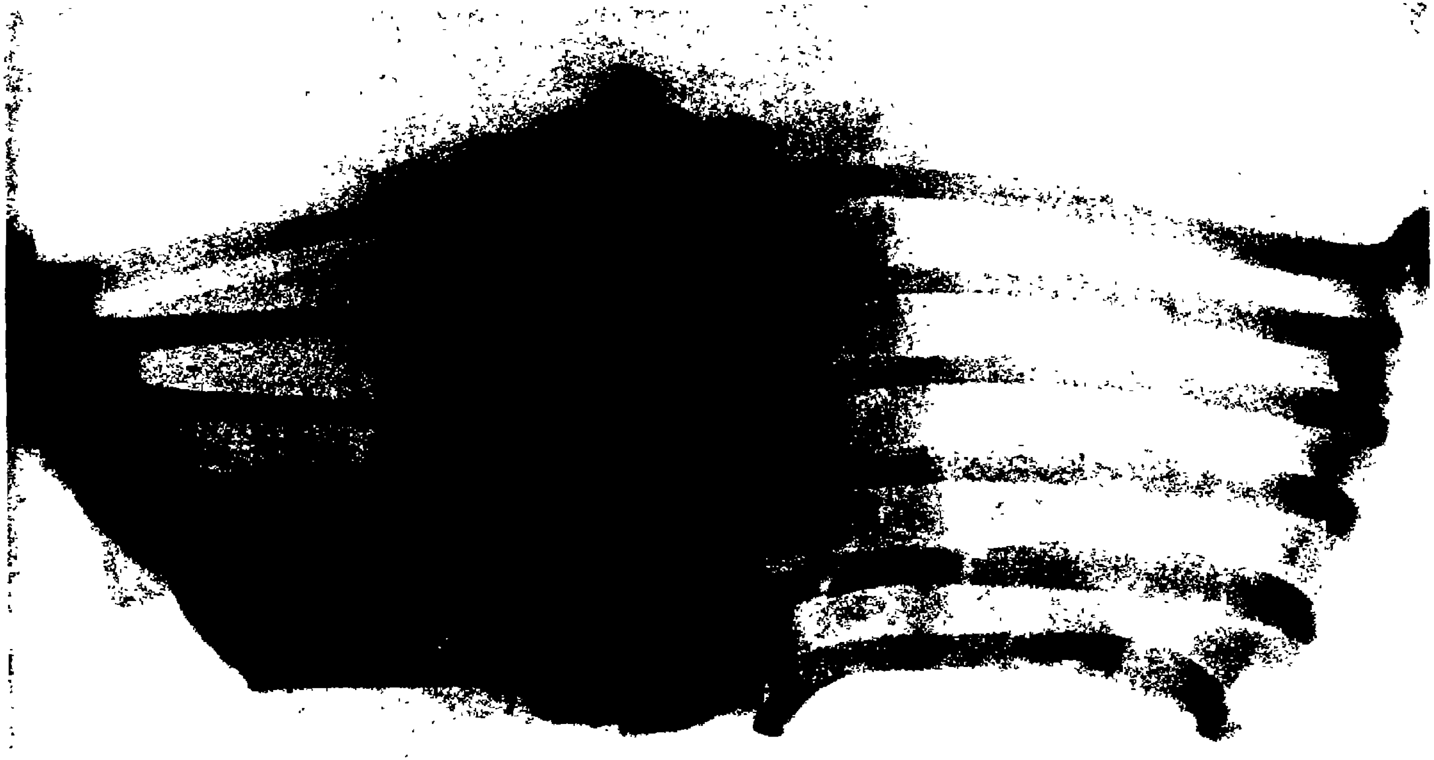


Fig. 3.

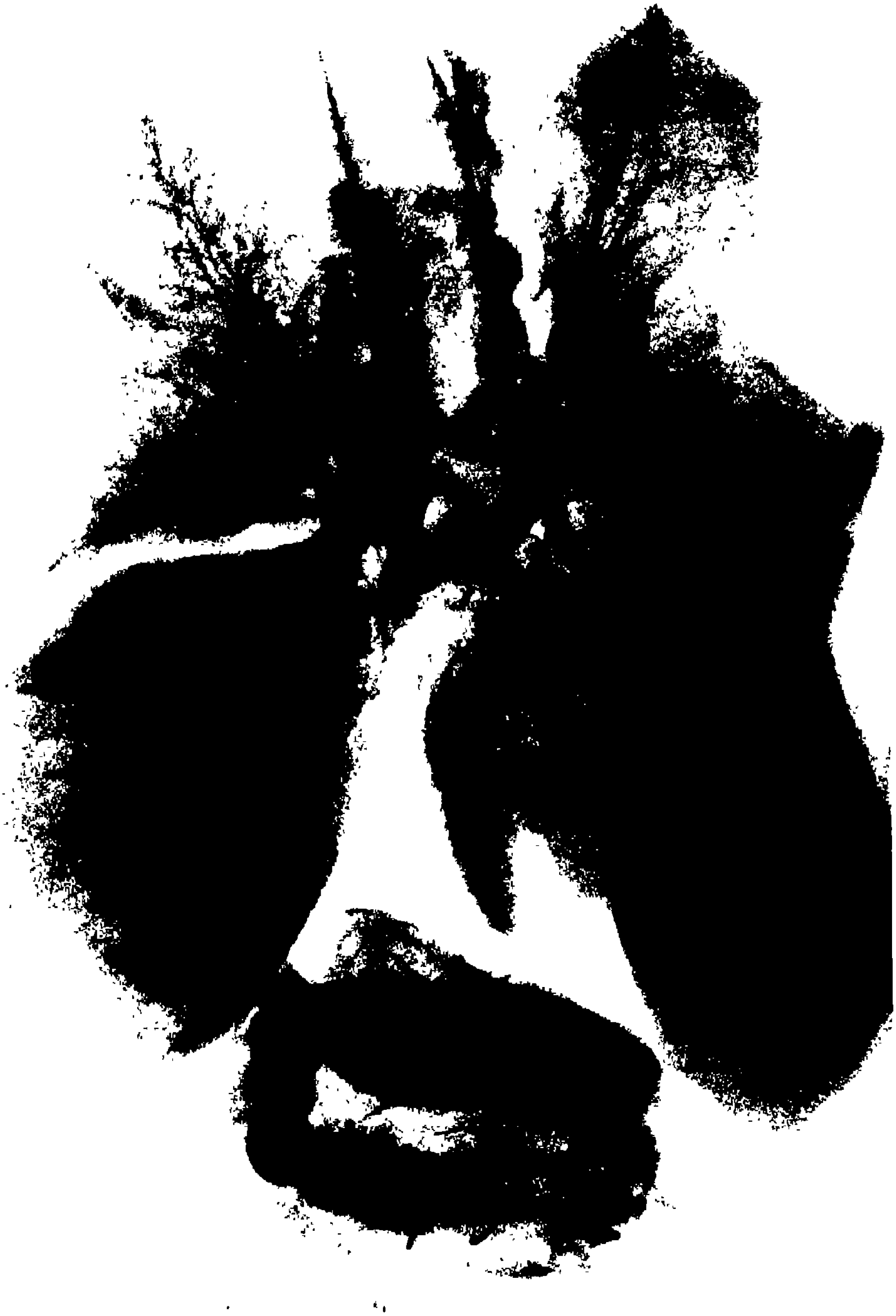


FIG. 3A.

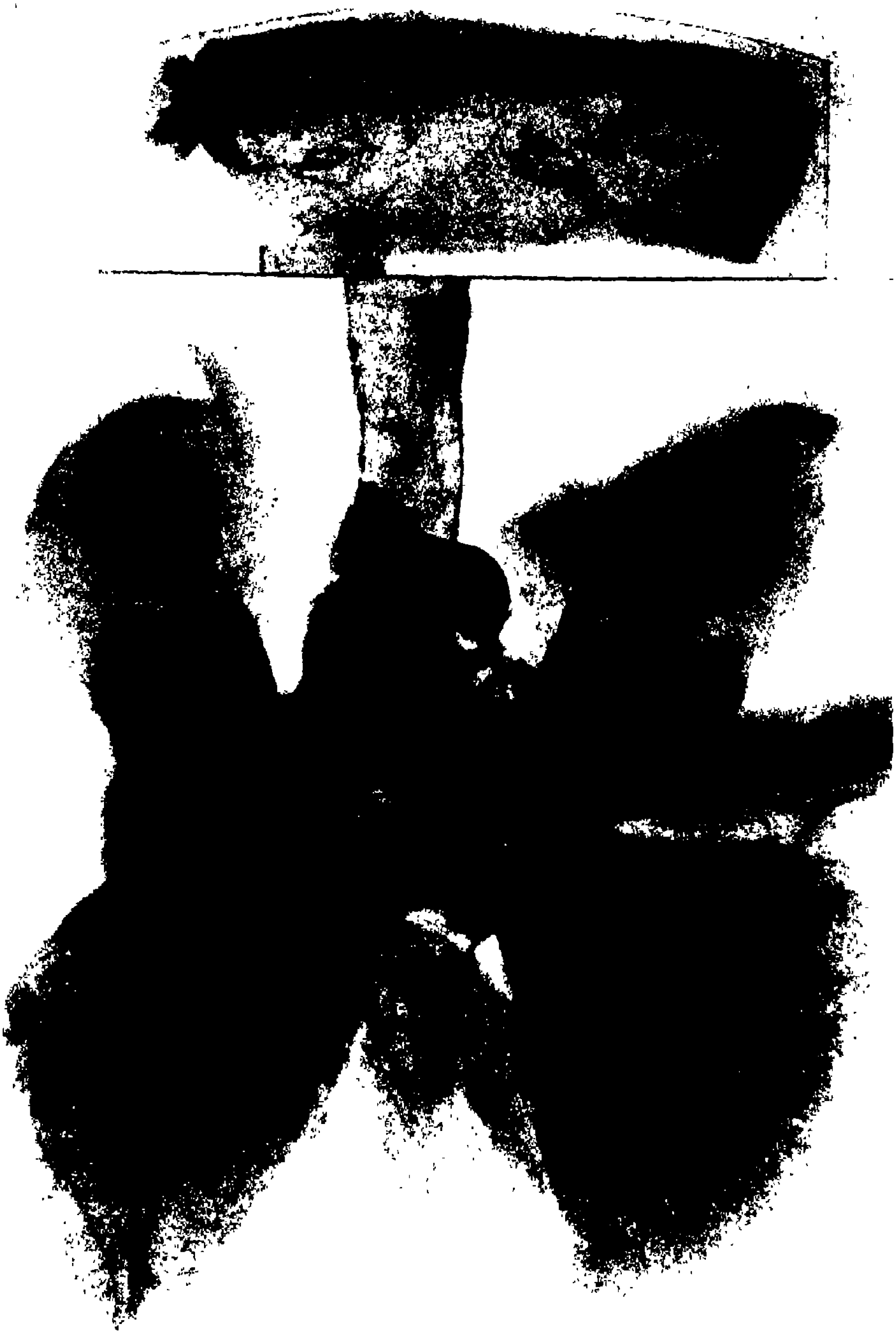


Fig. 4.

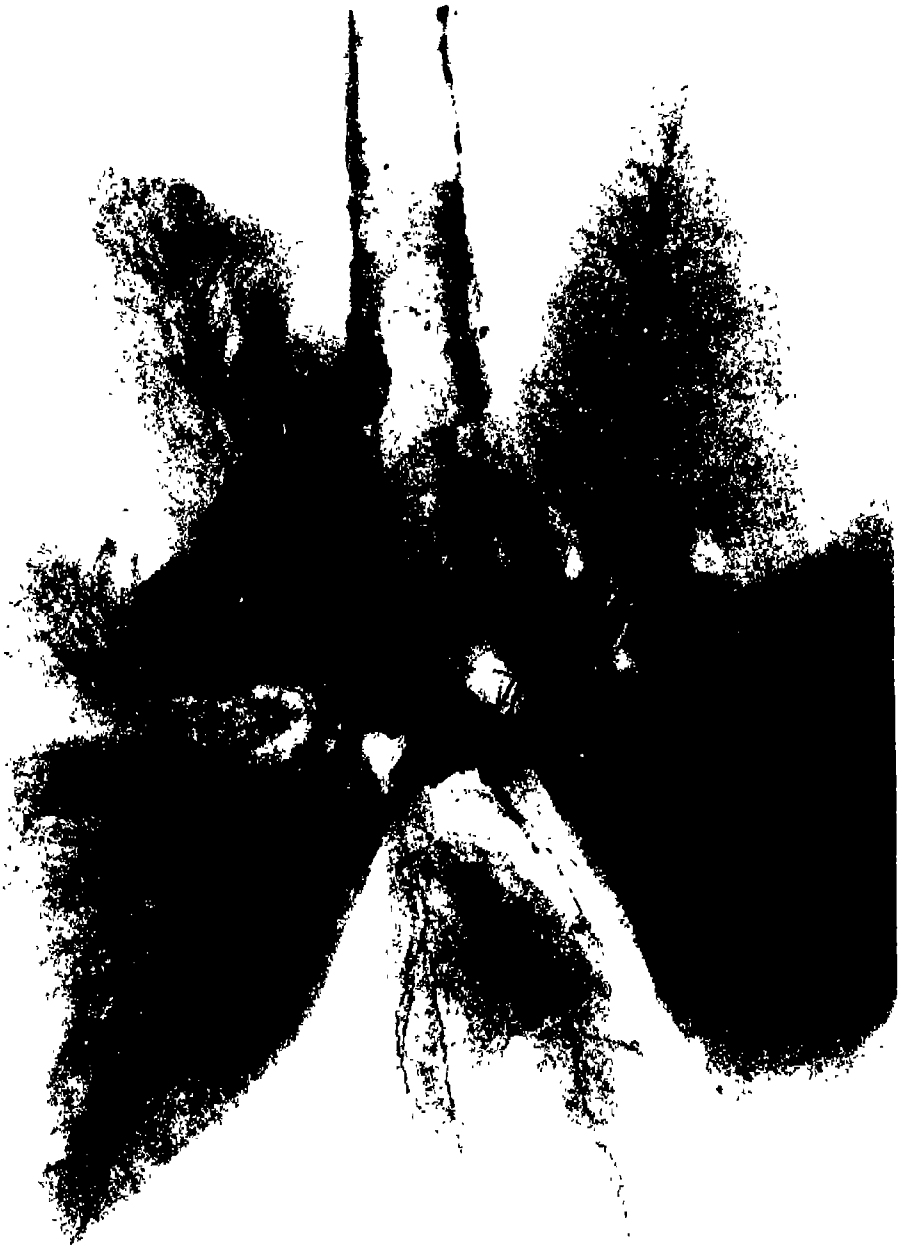


FIG. 5.

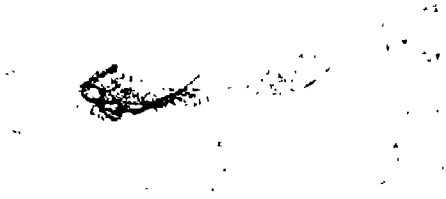


FIG. 8.

FIG. 9.

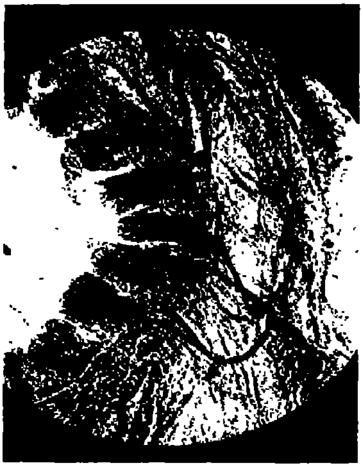


FIG. 10.

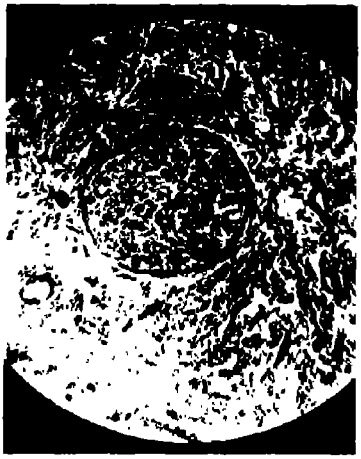


FIG. 11.

FIG. 12.

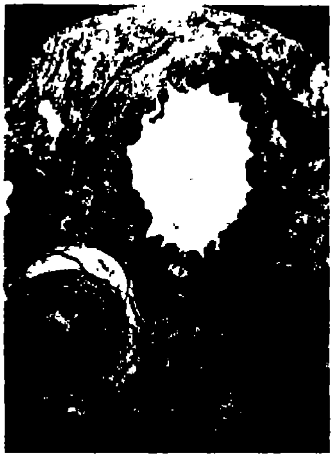


FIG. 13.

612 . 172 : 595 . 384 . 2

A Study of the Crustacean Heart Muscle.

By J. J. Izquierdo (Mexico).

(Communicated by E. D. Adrian, F.R.S.—Received January 2, 1931—Revised August 5, 1931.)

(From the Marine Biological Laboratory, Plymouth.)

It is remarkable that although nearly a century has passed since the attention of physiologists was first attracted to the crustacean heart (Weber brothers, 1846), the available data which have accumulated on its activities are very scanty. It was with the aim of acquiring wider and more accurate information that the present investigation was undertaken on *Maia squinado.*

METHODS.

The Heart Perfused in situ.

Inspection of the exposed heart of *Maia* shows at once why it collapses if the tension, to which the organ is submitted in its normal position, is suppressed by severing in turn its radiating alæ. The organ, thus excised, cannot be suspended from one of its arteries because these are not resistant enough. Therefore, for establishing connection with the recording lever, ligatures at the anterior and posterior segments are necessary. Of these one can be made so as to include the cannula for perfusion.

Frequently the heart of *Maia* has been merely exposed and moistened from time to time with a few drops of sea-water (Carlson, 1906; Hoffmann, 1911; Polimanti, 1913; Mangold, 1924–25; de Boer, 1928). But from the irregularities in the tracings published, or the definite statements made by the authors, it is clear that the heart soon gets into a bad condition. Even when more or less long series of regular beats are found, anomalous beats occur (see fig. 1), *i.e.*, " fractioned systoles " (de Boer) which, as the " wiry " slow beats described by McWilliam (1918) in the damaged mammalian heart, testify to the abnormal character of the conduction. The considerable slowing of the exposed and empty hearts also constitutes evidence against the use of this method. For the present research the heart has been always perfused *in situ.* The method used is as follows :—

The limbs having been removed, the heart or " ventricle " was easily exposed by excising a large circular portion of the chitinous carapace covering the cardiac region. In doing this, care was taken not to damage the subjacent pigmented dermis and pericardium. The whole body of the *Maia* was then tied down upon a heavy round plate; the pericardial roof dissected off, and the pericardial cavity flushed out with the perfusing fluid.

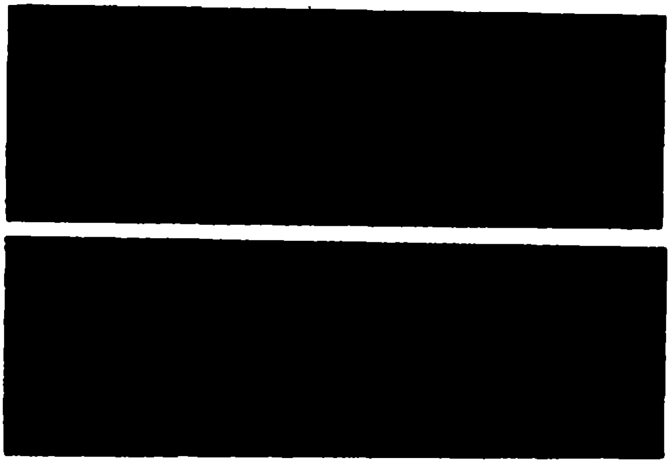


FIG. 1.—"Fractioned" or "wiry" beats resulting from impaired conduction in exposed *Maia* hearts, left empty without perfusion. Time intervals in seconds.

An incision was made on the middle-line of the posterior part of the ventral sheet of the pericardium and through it a ligature was passed round the superior abdominal or posterior aorta and used to tie in position a glass cannula in relation with the perfusion apparatus (fig. 2).

It is often pointed out that sea-water, although having approximately the same osmotic pressure as crustacean blood, contains about six times more magnesium (MacCallum, 1910). This notwithstanding, it has been repeatedly ascertained that the heart of a large number of decapod crustaceans continues to beat in this fluid (Carlson, 1906; Polimanti, 1913; Hogben, 1925; Bain, 1929) and that the nerves of *Maia* will keep alive and in good condition in it for many hours (Levin, 1927). Neutralised to a proper p_H, Mines (1912) found in it an excellent perfusion fluid for the hearts of some elasmobranchs. Other fluids which have been proposed, containing only Na and Ca ions (Hogben, 1925) are inferior to sea-water in that they are not buffered. According to this author they are able to maintain the rhythm only for 2 hours or more, and after the first half hour of perfusion it becomes extremely difficult to reproduce the effects resulting from ionic changes, as observed in the fresh heart.

If due attention is paid to pressure, sea-water indeed proves to be a satisfactory medium for keeping the hearts beating in such good condition that

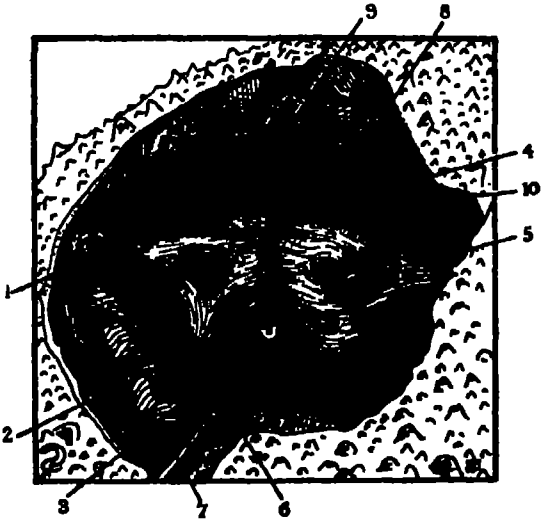


FIG. 2.—Dorsal view of the heart of *Maia* exposed and prepared for perfusion (semi-schematic). 1, antero-lateral ala; 2, postero-lateral ala; 3, posterior ala (sectioned); 4 and 5, anterior and posterior ostia; 6, superior abdominal artery with 7, cannula in position; 8 and 9, antennary and ophthalmic arteries; 10, point where the hook of the recording lever was attached.

many of the effects obtained are reproducible for many hours, even more than 10. No attempt was, however, made to find out for how long they would have kept beating.

Sea-water, from outside the breakwater of Plymouth Sound or from the tanks of the laboratory, was sometimes used in its original state (p_H about 8·2) for perfusion, but more frequently it was previously brought to the range of neutrality by carefully adding diluted HCl. Where special hydrogen-ion concentrations were required, diluted HCl or NaOH was added to obtain the desired p_H, which was roughly determined by using Clark and Lubs series of indicators and standard chart (Clark, 1922). When a greater decrease in the hydrogen-ion concentration than the one covered by these indicators was required—namely, to about p_H 10·2—first the NaOH solution was added till a persistent magnesium precipitate began to appear, and the same amount again to reach the middle of the step in Haas titration curve (Haas, 1916). The p_H was then checked against phenolphthalein and alizarin yellow G standards (Atkins and Pantin, 1926). Samples of the outflowing fluid from the cannula were taken at the start and after the end of each experiment, which usually gave the same p_H. Before each experiment a current of air was kept bubbling through the sea-water. During the experiment, the air bubbling through the tube of the Mariotte bottle—which in addition served to regulate the pressure and flow of perfusion—also kept the fluid aerated and constantly mixed. The pressure was regulated (at about 100 to 120 mm. sea-water) by adjusting the screw-clamp on the rubber tube leading from the Mariotte bottle, or by changing the height of the Mariotte bottle or of the tube in its interior. All experiments were made at room temperature (10° to 16° C.), kept fairly constant during each one of them. About 80 preparations were used, during the months of September and October, middle-sized hard-shelled specimens, which gave the better results, being preferred.

As there is evidence showing that perfused organs do not come into equilibrium with their new environment before about half-an-hour, no experiment was made before perfusion had been going for this time.

The Anterior Segment Preparation.

A quiescent preparation of the ventricle is easily obtained in the lower vertebrates by making a first Stannius ligature. In *Maia* heart, in which we do not know with certainty where the beat originates,* this is not applicable. A few attempts to get information about this point showed that, after making the most varied sections upon the heart, every one of its segments was seen to beat spontaneously, especially if perfused with alkaline fluids or put under the tension of the recording lever. The method which finally was found to give better results consisted in making a complete transverse circular cut passing just in front of the postero-dorsal pair of ostia which separated the heart into two halves. The anterior one, which will be called in this paper the anterior segment preparation, often remained quiescent sufficiently long for experiments which needed the elimination of the spontaneous beat to be carried out. Occasionally the posterior segment served better for this purpose.

Recording and Stimulating Arrangements.

For the earlier experiments a light isotonic lever, amplifying the movements about 10 times, was used.† Subsequently it was replaced by a calibrated torsion-wire isometric lever. The least possible initial tension compatible with the securing of good records was used, generally from 2·0 to 3·5 g.; more exceptionally below these values, to 1·3 or above them to 5·0 g. In either of these cases the lever was attached to the heart by means of a fine fish-hook.

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* For hearts in which conduction is abnormal, it has been reported to take origin in the anterior segment (Mangold, 1925, *b*).

† The slight kick noticeable in some of the isotonic records, at the end of the systolic ascent, is due to the inertia of this lever.

To record the contractions of the ventricle perfused *in situ*, this hook was generally fixed in the position shown in fig. 2, midway between the dorsal antero- and postero-lateral alæ, but anchored not on the very border of the organ but well below it, almost on the ventral cardiac aspect. In this way the systoles were recorded as upward movements. It should be noted that under these conditions, not only the movements of the segment between the neighbouring insertions were recorded, but also those from other parts of the musculature, which were transmitted to all the points of the cardiac walls by the fluid filling the organ.

The contractions of the anterior-segment preparation were recorded with the hook fixed more or less on a middle point of the sectioned dorsal aspect. By doing this, a fairly uniform tension was applied to the segment, which prevented it from collapsing.

For stimulation, break induction-shocks were exclusively used, from one or two induction coils. The make-shock was short-circuited by using a Palmer's rotating contact-breaker.

For recording only the moment at which break-shocks were applied, a modified form of an ordinary electromagnetic signal, included in the primary circuit of the coil, was used (see fig. 3).

The faradic stimuli were always applied by the monopolar method. The small hook of the recording lever was connected with one of the terminals of the secondary by means of a fine insulated copper wire, and was used as an

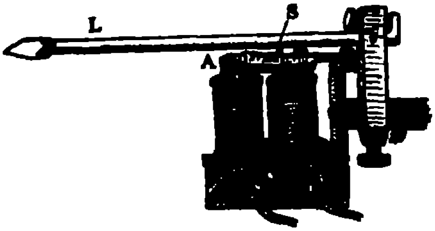

FIG. 3.—Ordinary time-marker modified for registering only the breaks in the current. The writing point is replaced by a recording lever L, the position of which is unaffected when the armature A is drawn at the make, and is changed only when it overswings through its resting position, at the break. The upper edge of the slider S, firmly fixed to A, just touches the lever above.

active electrode. A small silver wire connected to the other terminal served as an indifferent electrode.

Thus, the contractions were recorded exactly at the point at which the stimuli were applied. To have a greater concentration of current at this point, it was often found more convenient to keep the point at which the hook was attached well above the surface of the fluid outside the heart.

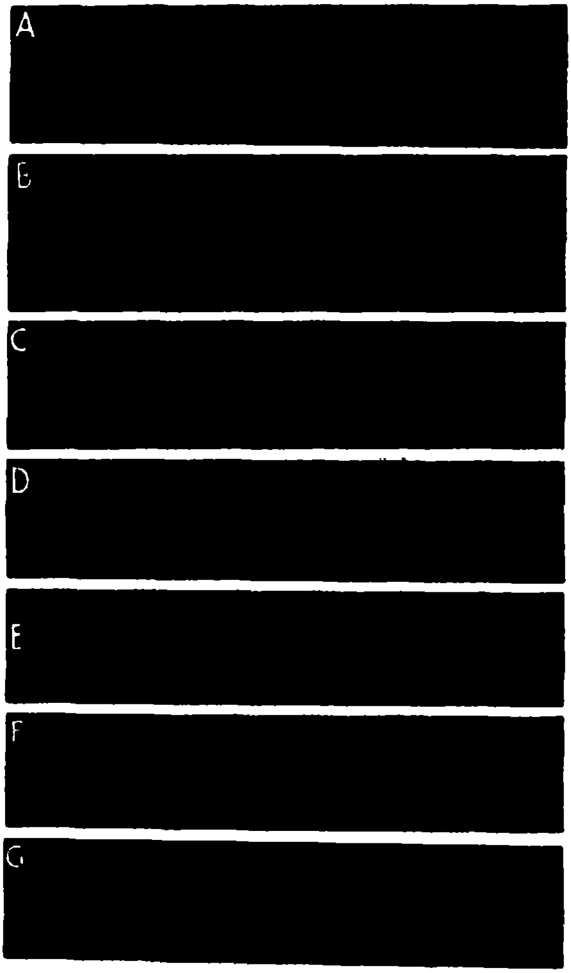

Fig. 4.—Isotonic records of the natural heart beat of *Maia* with varying perfusion pressures. A was taken when the heart was empty, and sections B to G, when pressure was 11, 58, 177, 232, 570 and 764 mm. of sea-water, respectively. Time intervals in seconds.

RESULTS.

Effects of the Perfusion Pressure upon the Frequency of the Heart.

The influence of pressure has been noticed before by Carlson (1906, *a*) and by Mangold (1924, *a*), who noticed that the exposed *Maia* heart beats very slowly when empty, and more frequently and regularly when filled, but no numerical relations were established. Perfusion alone is not enough to maintain the heart beating regularly and at " normal " rate. If the perfusion is made under pressure of only a few millimetres of sea-water, after a sufficient time the rate of beating slows down considerably to about 8 to 12 beats per minute and abnormal beats are seen among the normal ones. If then the pressure is gradually raised at regular intervals of half to 1 minute, as the ventricular volume becomes bigger and bigger, the frequency of its natural rhythm is also observed to increase (see tracings in fig. 4). Up to a pressure of 100 to 150 mm. of sea-water the increase is so rapid that the frequency becomes two to seven times greater than the initial one (see curves A, B and C in fig. 5). It should be noted that this range of pressure agrees with the figure of 12 cm. of water given by Brücke and Satake (1913) as the value of the arterial pressure of a close related species (lobster). From that limit, with further increase of

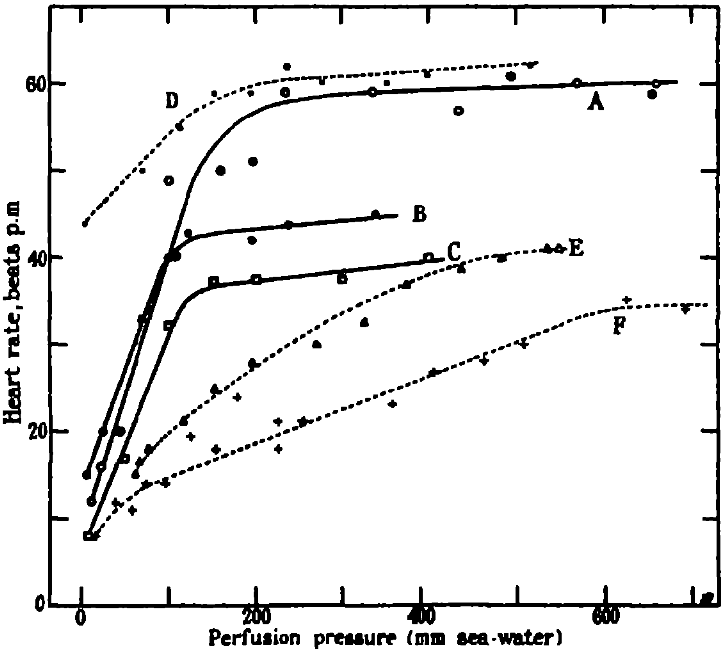

FIG. 5.—Relation between frequency of heart beats and perfusion pressure (see text).

pressure, the frequency rises much more slowly and beyond 600 mm. it may rather decrease. Another type of response to the rise of pressure is presented in curves E and F of fig. 5. The rate of beating rises and the step is only reached when the pressure has attained 400 to 600 mm. This was the type of response obtained when the pressure effect was tested upon hearts which were still contracting at a rapid rate after the perfusion pressure has been already reduced (curve D, fig. 5).

It would be desirable, therefore, to investigate the relation between the systolic energy of contraction and the length of the fibres of *Maia* heart (volume). This is still more interesting because it seems that in *Maia*, venous pressure cannot be the factor determining the initial filling of the ventricle as in the mammalian and frog's heart.

The All-or-none Law.

New evidence about the way in which the magnitude of the contractile response of the crustacean heart changes with variations in the intensity of the stimulus is needed, since the results of Hunt, Bookmann, and Tierney (1897) were contradicted by Carlson (1906, *c*). The increasing height of contractions with increasing strength of stimulus, observed by Hunt and collaborators, was considered by Carlson as only obtainable from greatly fatigued preparations, but not in preparations in which excitability and conductivity are only slightly impaired. These, he reports, "will respond with a contraction of uniform strength to stimuli of increasing intensity within a wide range."

Fresh anterior segments prepared after the end of half-an-hour perfusion with neutral sea-water were used. They were stimulated at regular intervals with break-shocks from a Harvard induction coil with no iron core. At the beginning of each experiment the secondary was moved up to the position where the break-shocks would just evoke a response and left without altering this preliminary setting during the whole experiment. As in a coreless coil the intensity of the current in the secondary is proportional to that in the primary, which was progressively increased by removing resistances in it, the primary current was measured with a Crompton amperemeter and used to express the intensities of the faradic shocks. A series of isometric responses from one such experiment is reproduced in fig. 6. For every group of five stimuli of the same intensity, the current strength is indicated below the intermediate marks. As the intensity of the shock is very gradually increased, the response grows only in *steps*, sometimes of one size, sometimes of another, with the limitation that for each group of similar stimuli there is certain progressive alteration in the sizes (tension) of the beat, due, as will be shown later, to the improving effect of repeated stimulation. The bigger increase at the beginning of each group is clearly the result of the longer pause preceding it. The steps reach finally a perfect definite value—in fig. 7 they have been plotted in percentages of the maximal response—beyond which stronger shocks rather produce a decrease in the response, in a manner similar to that observed by Fröhlich in frog's muscles. Coincident results were obtained by reversing the order of the experiment.

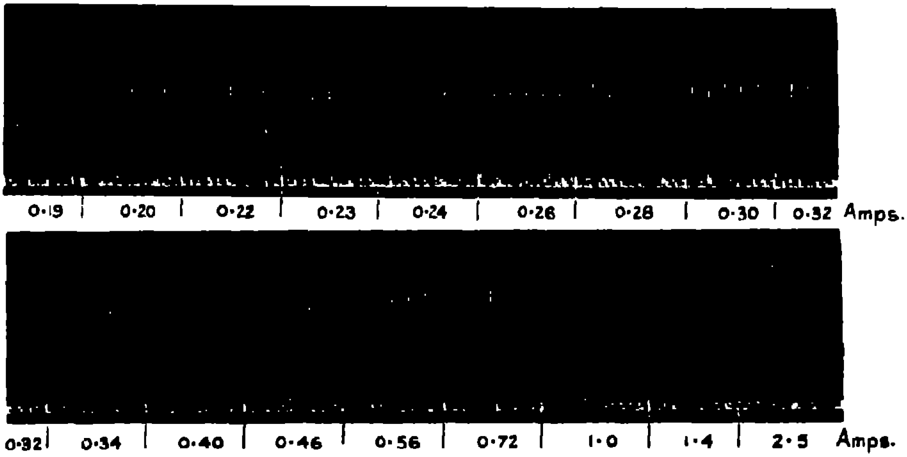

FIG. 6.—Isometric response of *Maia* heart (anterior-segment preparation) to break-shocks of increasing strength from a coreless coil, with its secondary kept at 3 cm. distance throughout the experiment. The intensity of the primary current is increased at each big mark and kept constant at the values written between each two of them. Preparations made after 40 minutes perfusion with sea-water p_H 7·1 at 11·8° C. Time marked in 1-second and 10-second intervals. The lower record is an immediate continuation of the upper one.

In view of well-known evidence (Lucas, 1905–1907; Mines, 1913; Pratt, 1917; Hartree and Hill, 1921) the explanation of these results is that they are due to a continuously increasing number of muscle fibres, indirectly or directly stimulated. The heart of *Maia* deviates in this respect from the frog's heart (Bowditch, 1871). It is more similar to the skeletal muscle of vertebrates, in that conduction does not spread over the whole heart.

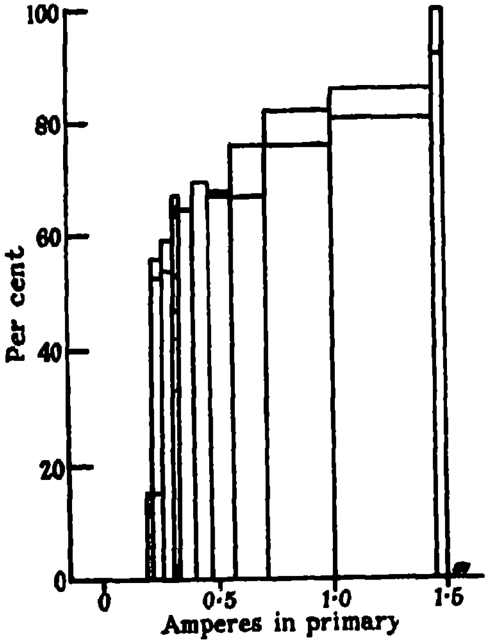

FIG. 7.—Relation between the maximum tension developed in the response of *Maia* heart and the strength of the break-shocks evoking it.

Nature of Contraction of Maia *Heart.*

The only published electrocardiograms of *Maia* are those that Hoffmann (1911) obtained by leading from electrodes placed upon exposed and empty hearts which, as I have demonstrated, are under very abnormal conditions. What is said above is enough for interpreting them as forms of "dissociated systoles." The suspicion about their abnormal character grows stronger after considering that the features of the electrocardiogram recorded by Clark (1927) in a closely related species (lobster) are the same as observed in the lower vertebrates. Nevertheless, the conclusion based on those tracings, that the normal contraction of *Maia* heart should be considered as a tetanus, still prevails in physiological literature (see Clark, 1927).

Apparatus for taking an electrocardiogram of *Maia* was not available at Plymouth. Some, however, of the experimental evidence to be dealt with in the next two sections of this paper, is very much against the tetanic nature of the normal contraction of *Maia* heart. The refractory period, covering the whole heart (*a*) was always found to be restricted to the beginning of the rise of tension, and (*b*) was immediately followed by a period during which the heart responded with contractions of progressively growing size. These phenomena are inconsistent with the idea of a tetanus.

The Refractory Period.

As a fair number of investigators—a list is given in de Boer's paper—beginning with the brothers Weber (1846), have continually reported that the contraction of the crustacean heart is not followed by a period of refractoriness, it is not surprising to find that this view is still the one prevailing in current physiological literature. Only Carlson (1906, *b*) suspected a condition of greatly reduced excitability, which he considered of an inferior degree to the one observed in the vertebrate heart, and de Boer (1928), more recently, reported that *Maia* heart is in a refractory state from the beginning to the end of systole.

In investigating this point I have made, upon curves from 34 different preparations perfused at p_H 7·0 to 8·2, measurements of the intervals after which a strong break-shock, well above threshold, falling after a previous similar one—

(*a*) fails to provoke a response (absolute refractory period);

(*b*) produces a response which just interrupts the path of the curve, probably because it is not propagated (relative refractory period); and

(*c*) begins always to produce a response (period of responsiveness).

The intervals were measured from the two marks of the signal magnet showing the moments at which the two break-shocks are sent in. Either the whole heart, or the anterior segment preparation, was driven by rhythmic break-shocks, and another extra shock was made to fall every few beats at different moments of the cycle. A curve from one of these experiments, from a heart driven at 0·82 second intervals, is reproduced in fig. 8. The first four extra stimuli fall 0·20, 0·34, 0·36 and 0·18 second after a rhythmic shock

FIG. 8.—*Maia* heart driven at 0·82-second intervals. An additional extra shock is thrown in at different moments of the cycle for the purpose of measuring the refractory period. Perfusion pressure 128 mm. sea-water; p_H 8·1; 15·8° C. Time in seconds.

and produce a response; the fifth, coming after only 0·16 second, falls within the refractory period and produces none. One hundred measurements, including only the maximal intervals for no response, obtained from eight different hearts, are plotted in fig. 9. The close coincidence of the two maxima of distribution of the maximal intervals for no response and the least intervals for an incipient response give 0·165 second as the average limit between the absolute and relative refractory periods. The distribution curve of the least intervals for full response reaches its maximum at 0·205 second. This may be taken as the end of the relative refractory period—therefore of about 0·04 second duration—and the beginning of that of full responsiveness. The total period of refractoriness is about 0·2 second.

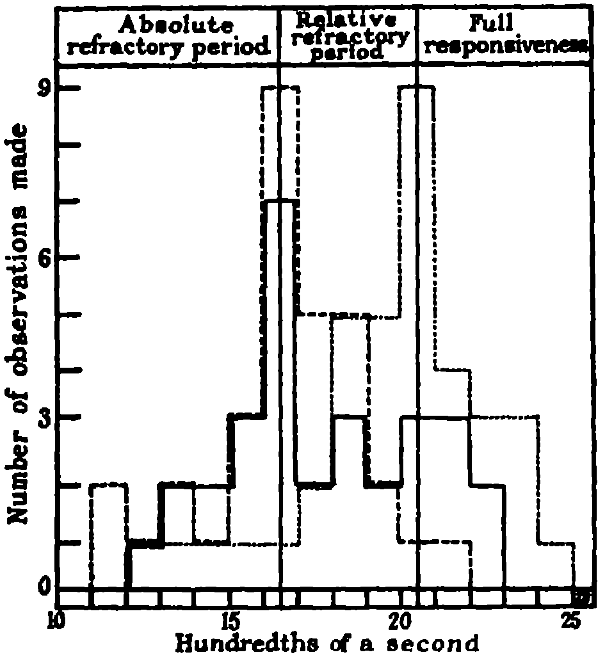

Fig. 9.—Distribution curves upon the same time-scale of 100 measurements from eight different hearts, for maximal intervals for no response (broken line) and least intervals for localised (continuous line), and full response (dotted line). All observations made between 14·8° and 61·5° C.; p_H 7·0–8·1, and perfusion pressure 80–128 mm. sea-water.

A certain error must be admitted in these measurements, due to the fact that they were taken irrespectively of their position in the cardiac cycle. But this cannot be great, because other measurements obtained by pairs of shocks given to anterior segment preparations every 10 seconds (see records in fig. 12) gave essentially the same results.

Applying these last measurements to one of the time-tension curves (see fig. 10), we arrive now at the new notion that the refractory period of *Maia* heart *represents about one-third of the time during which the tension rises and is kept high*. If the contraction is drawn out, then the fraction occupied is proportionally less.

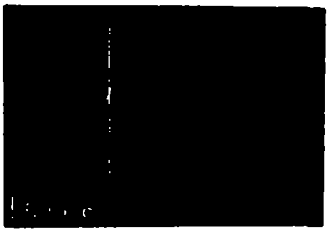

FIG. 10.—To illustrate the position and relations of the refractory period (white line) within a single heart contraction. The curve is taken isometrically upon a quickly revolving drum. Time intervals, one-fifth second.

Maia heart is therefore far from having the extraordinary long refractory period which in the vertebrate heart overlaps the time of maintained tension. Compared with frog's skeletal muscle, however, its refractory period is notably longer.

Compensatory Pause.

A direct consequence of the existence of a refractory phase in a rhythmically contracting heart is the production of the so-called "compensatory pause." The necessary condition for this is that an extra shock should be made to precede one of the rhythmic stimuli—natural or faradic, this is immaterial—originating the beat by an interval slightly shorter than the refractory period. This being long in the frog's heart, the effect is easy to obtain with relatively long intervals. In *Maia* it is evident, on the one hand, that the extra shock must be thrown in with an anticipation of less than one-fifth of a second; on the other, that the slower the rhythm the more inappreciable will appear the shortening and enlarging of the cycles before and after it. Relatively long compensatory pauses are only obtained when the heart is driven at such a rapid rhythm that the refractory period covers a good part of each cycle. For example (see fig. 11) in a heart driven at 0·3 second intervals—consequently in undulatory tetanus—after an extra shock falling 0·16 second after the rhythmic stimulus 2 produces a contraction E, the next rhythmic shock 3, coming 0·14 seconds later, finds the heart irresponsive. The compensatory pause of 0·44 second following the extra shock is accompanied by a notable fall of the writing lever. The beat following is *larger than the others because the muscle has had a longer rest.* The duration of the shortened period and the compensatory pause, added together, are equal to that of two rhythmic periods. The analogies with the Marey's phenomenon are complete (Marey, 1875).

Fig. 11.—To illustrate conditions under which compensatory pauses are demonstrable in the most conspicuous way. The heart was driven at 0·32-second intervals, while perfused under pressure of 128 mm. sea-water, p_H 8·1, temperature 15·9° C. The extra shock between stimuli 2 and 3 produces an extra beat E which is followed by a compensatory pause. The beat following shock 4 is relatively larger on account of the more prolonged rest. One-second intervals.

As de Boer (1928) used extra shocks, spaced at longer intervals from the rhythmic natural stimuli of the beat, this explains why he did not succeed in producing compensatory pauses.

Recovery after Contraction.

The gradual return of contractility after the end of the refractory period was closely followed by using essentially the method originated by Engelmann and used by Börnstein (1909) and Adrian (1920–1921) for the frog's heart and by Hartree and Hill (1921) for the frog's sartorius.

As it was necessary to avoid the disturbing influence of fresh spontaneous beats, quiescent anterior segments prepared after the routine half-hour perfusion, as described above, were exclusively used for the purpose. They were found to yield consistent results, at least during the first half-hour which followed. Usually it took only about 15 to 20 minutes to get a sufficient number of points for plotting a curve. Not rarely, points taken within an hour's time were found to fall upon the same curve, but especially after acid perfusion—we shall insist upon this immediately—the speed of recovery was found gradually to decrease as time passed.

As for the measurements of the refractory period, pairs of strong break-shocks, well above threshold to insure a response, were regularly sent in, at constantly increasing intervals (see fig. 12). In a first series of experiments intervals up to 30 seconds were used; but, as it was constantly found that after 5 or 6 seconds the size of the second contraction did not show any important variation, other than those due to the probable error of the method, in this particular application, ±5·0 per cent., 10 seconds was fixed as the

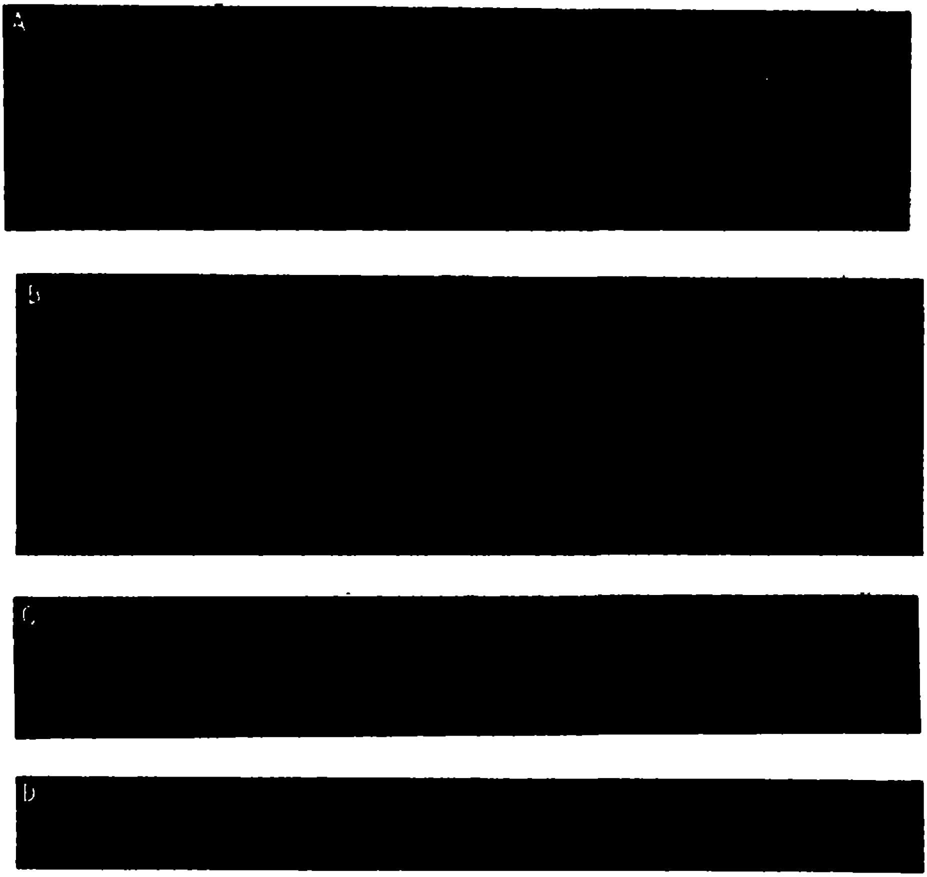

Fig. 12.—Records showing the variation in size (tension) of a second response of *Maia* heart to a maximal shock as this is thrown in at progressively longer intervals after a first one. Before making the anterior segment preparations required (see text), hearts A, B, C and D were perfused with sea-water of p_H 5·2, 6·3, 7·1 and 9·8, respectively, at about 14·0° ± 0·5° C., and 110 mm. sea-water pressure. Time intervals in seconds.

routine maximal interval for investigating the variation of the second response. Responses begin to occur at the earliest possible moment after the end of the refractory period, therefore well before the first mechanical response had developed. The tension added by the second response, as well as the exact moment at which its maximum occurred, were found by subtracting the ordinates of the tension curve of a first response, from those corresponding to a second one, as done by Hartree and Hill (1921).

Altogether the experiment has been repeated with good results in 30 different preparations perfused with sea-water of p_H ranging from 5·2 to 10·2. As it has been said that the heart of *Maia* " will stand " (Ritchie, 1928) or " still function well in a fluid with p_H 4·0 " (Clark, 1927), perfusions with such extremely acid fluids were tried, but were found unsatisfactory. Nine hearts perfused at p_H between 5·1 and 4·0 had to be rejected because they had lost their excitability even before the anterior segment had been prepared. Only two preparations out of six perfused between p_H 5·4 and 5·2 could be used for mapping out complete curves, because of the same decrease of excitability and the sluggishness of the contraction. So far, the extraordinary resistance of the crustacean heart towards very acid perfusion fluids has not been verified. The limiting acidity with which the ventricle of *Maia* can be in equilibrium without undergoing rapid changes is much about the same as observed for the frog's heart, viz., about p_H 6·2. But it is fair to add that Hogben's statement, which seems to be the basis for the views expressed above, was made for changes " detectable after 5 minutes' perfusion " (Hogben, 1925), a period of time after which it could hardly be admitted that any perfused heart is already in equilibrium with the medium (amongst much other evidence which could be quoted, see Izquierdo, 1929). Instead, how well the *Maia* heart stands perfusion of an extreme alkalinity, up to p_H 10·2, can be appreciated from the fact that only three preparations perfused between p_H 9·7 and 10·2 had to be rejected (out of eleven) and this was only because they went on beating continuously and were therefore useless for the experiment.

The set of curves reproduced in fig. 13 shows how, by subtracting the ordinates of the two double tension-curves for a single maximal shock (I) and for two maximal shocks (II), superposed, the third curve (D) giving the mechanical response produced by the second shock was constructed. By plotting the maximum tension produced by each second response against the corresponding interval between the two shocks, as in fig. 14, when the perfusion has been with neutral or alkaline sea-water, a gradually rising curve is obtained, with the general characters of the known recovery curves of excitable tissues.

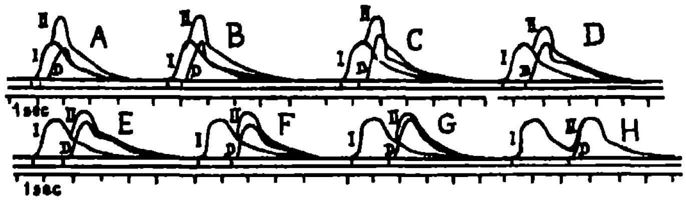

Fig. 13.—Superimposed isometric tracings of the responses to a single shock (I) and to a pair of maximal break-shocks (I and II) given at progressively increasing intervals. Curve D obtained by subtracting the height of the ordinates of I from the corresponding ones of II, represents the tension added by the second response. Before making the anterior segment preparation, the heart was perfused under a pressure of 10 mm. sea-water; p_H 6·3, 14·8° C.

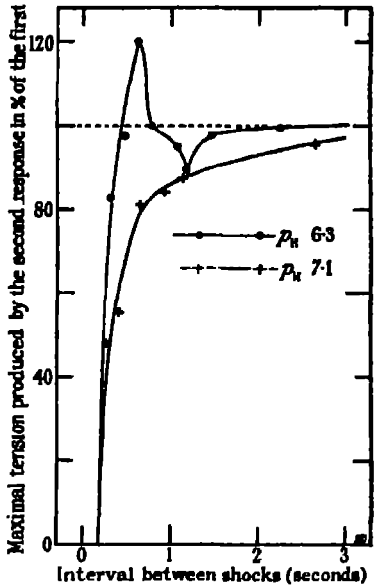

Fig. 14.—Relation between the tension developed by a second shock and the interval after which it follows the first one. The tension developed has been found from curves like those in fig. 13 (see text).

As a matter of fact the curve does not deviate very far from Adrian's curves for the frog's heart (Adrian, 1920–1921). The time relations are very close; within 3 to 4 seconds of a previous contraction a new response is already as big as the first one, except after perfusion with a very acid fluid at about p_H 5·4 in which case the normal value is reached only after 5 to 8 seconds. But the evolution of the curve beyond this point is somewhat different in *Maia* heart. The hearts, not only of the frog [as is evidenced by tracings of Bowditch] plotted by Mines (1913); of Adrian (1920); of Fiddes (1928) and many others, but also of elasmobranchs [as shown by records of Mines (1913)] from this stage pass through a supernormal phase, demonstrated by Adrian (1920–21) to be conditioned in the frog by an acid perfusion at about p_H 6·3. In 11 preparations of *Maia* hearts perfused with sea-water of p_H 8·3 to 10·2 no supernormal phase was observed, but the curve settled at once to the 100 per cent. value. After perfusion of 14 preparations with sea-water of p_H 6·2 to 6·4 the existence of a supernormal phase was very questionable and, at any rate, much less marked than in the frog's heart. This seems to constitute an important difference.

Summation of Contractions.

Carlson (1906, *b*) reported that a stimulus which reaches the crab's ventricle at the beginning of the systole, or rather " not at the very beginning," produces a superimposed beat. de Boer (1928) in a more recent series of experiments on *Maia* heart, found it impossible to produce summation. This must be due —as he says—to the methods he used, because, as would be expected from the time-relations between the contraction and the refractory period presented above, it will always be possible to obtain summation if the second stimulus is made to follow within, say, one- or two-fifths of a second after the refractory period. In other words, the least interval for summation slightly exceeds the value of the refractory period, and the maximum interval for summation is just shorter than the period of maintained tension. Furthermore, summation in the *Maia* heart seems to be favoured (*a*) by the fact that a twitch provoked even by a maximal faradic stimulus does not develop the maximal tension which it is capable of developing, and (*b*) by the shortening of the latent period of the second response. I have already said (see p. 239) that the distance between each pair of shocks was measured between the marks of the magnet-signal and not between the rising limbs of the two responses, because within certain ranges of time these appeared to be shorter. The three sets of figures below are illustrative of the changes observed with remarkable constancy in all the tracings (the same recording system being used) :—

1. *Maia* Heart Perfused with Sea-water of p_H 6·3.

Intervals between the marks	0·35	0·47	0·76	0·98	1·04	1·62	1·96	2·63	3·08	3·36
Intervals between the beginning of the responses	0·18	0·28	0·55	0·75	0·83	1·55	1·89	2·55	3·0	3·30

2. *Maia* Heart Perfused with Sea-water of p_H 7·1.

Intervals between the marks	0·24	0·34	0·55	0·92	1·08	1·20	1·78	2·58	3·24	3·37
Intervals between the beginning of the responses	0·17	0·21	0·38	0·81	0·93	1·12	1·66	2·51	3·17	3·30

3. *Maia* Heart Perfused with Sea-water of p_H 10·2.

Intervals between the marks	0·38	0·40	0·57	0·84	1·25	1·76	2·08	2·36	3·07	3·29
Intervals between the beginning of the responses	0·28	0·32	0·50	0·77	1·14	1·71	2·04	2·33	3·04	3·22

The effect is clearly greater the shorter the intervals considered, and it is very interesting to discover that for intervals longer than approximately 3·3 seconds the effect has disappeared and the two measurements are found to coincide. This close coincidence in time with the recovery curve indicates that it must be related to the effects of a previous stimulation, which probably facilitates the speed of propagation of the excitatory process and quicker development of tension.

The classical view with relation to the frog's heart or other excitable tissues has been that the latent period of a second response is always longer than that of a previous one. Trendelenburg (1911) questioned some time ago the long latencies reported by Lucas (1913), and Fiddes (1928) has insisted that the second mechanical response can be preceded by a latency as short as, if not shorter than, the first one; and especially that of some of the very long latencies figured by Marey (1875) are obviously erroneous. In the present case, in view of the facts reported, no other conclusion seems possible than the one given.

It should be noted that in hearts in which conduction has been dissociated, the "fractioned systoles" represent summated contractions of special type, which will not be analysed here.

Tetanus.

Physiological literature has for a long time contained references to the tetanus of *Maia* heart, but these have been confined to the statement that by faradisation a tetanus can be produced (a list of authors is quoted by de Boer) which can rise higher or to the same level as an ordinary contraction (de Boer, 1928).

If new stimuli are sent in at successive regular intervals of the length needed for summation (see above) up to the third or fourth, new successive steps of summation are produced which, if continued, produce a tetanus (see records B to E in fig. 15).

In the normal conditions of perfusion with neutral fluids, with stimuli at 1·8 to 0·4-second intervals, steps for summation are clearly separated and the ensuing tetanus is undulatory (see curves B and C in fig. 15). With intervals of 0·20 to 0·26 of a second, most frequently (see record D in fig. 15), but not always, complete fusion of the contractions is obtained. This last result is always secured when the faradic shocks are sent in at 0·08 to 0·12-second intervals (see record E in fig. 15), which were the smallest used for this investigation.

As the experimental rhythm is gradually quickened no point is reached in the series at which an abrupt drop to a half-rhythm is produced, as in the vertebrate heart (see Mines, 1913). The only constant result obtained is a more and more complete fusion of contractions, resulting from the more and more protracted character of each one of its elements. This is again an

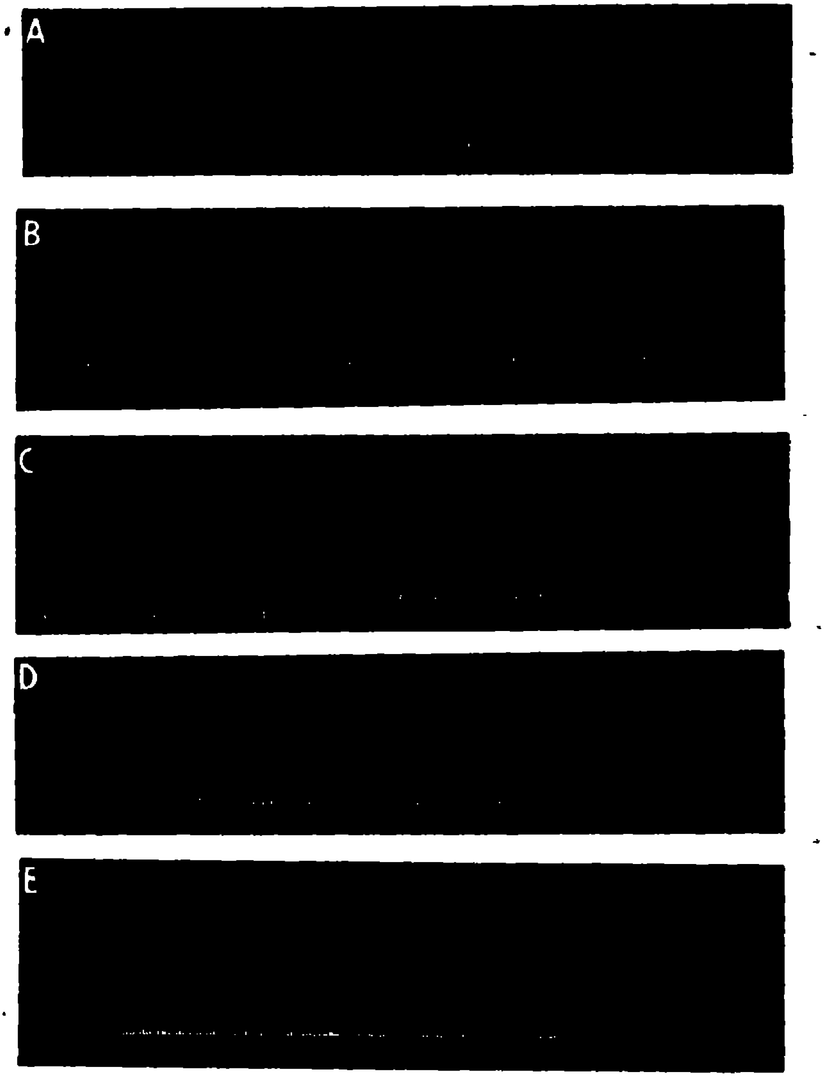

FIG. 15.—To illustrate the effects of high rates of stimulation. All the tracings, except B, are taken from the same *Maia* heart. Both hearts were perfused under pressure of 120 mm. sea-water; p_H 8·1; at about 14·5° C. A, B, C, D, E were driven at 0·8, 0·4, 0·3, 0·26 and 0·10-second intervals respectively (see text). Time intervals in seconds.

important difference explained by the relative shortness of the refractory period.*

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* After perfusion with an acid fluid at about p_H 7·0–4·0 the contractions are so sluggish that tetanus and very nearly fusion of contractions are observed at intervals as long as 1·7 second.

Effects of Renewed Activity—Staircase Effect.

The first response—either of the whole beating heart or the quiescent anterior segment—to a regular series of break-shocks thrown in at smaller intervals than those required for the complete relaxation of tension, has a size depending on its position in the recovery curve. But the next and following ones often grow larger and larger, in a *staircase arrangement* (see record A in fig. 15), well known in skeletal muscle. The same is still seen when the intervals are so short as to produce an undulatory tetanus (see record B in fig. 15).

Often the effect is merely an artefact produced by fatigue (*cf.* Fröhlich, 1905); the first stimulus after a pause finds the muscle rested and the contraction and relaxation are rapid; after a few stimuli the onset of fatigue prolongs the rate of relaxation without at first diminishing the force of contraction. But also, frequently after an initial period of this type (see record A and B in fig. 15) the size of the contraction begins to increase and to exhibit at the same time indications of increased "efficiency"; steeper ascent, more rapid and complete relaxation, beginning of the next beat from a more relaxed condition, and very probably decrease of the refractory period. I have said before that to produce complete tetanus, it is sometimes necessary to repeat the shocks at smaller intervals than those measuring the refractory period of the heart beating at slower rates. At quicker rates the effect was especially notable, as in fig. 15, B.

Summary.

The following new experimental evidence was secured with relation to the heart of *Maia squinado.*

When the perfusion pressure is gradually raised, the frequency of the natural rhythm also rises in accordance with either one of two types of curve.

The limiting acidity with which the heart can be in equilibrium without undergoing important changes is of about p_H 6·2. Thus far, the statement that it can resist greater acidity was not confirmed.

Break-shocks of gradually increasing strength evoke responses growing in a step-like arrangement towards a definite maximum.

After an effective stimulation the heart passes through a period of total refractoriness of about 0·2 of a second, which only occupies approximately one-third of the time during which tension is maintained. This, together with the values found for the general characteristics of the contraction and the possibility of evoking summated contractions which follow a definite law, are much against the tetanic nature of the contraction.

Compensatory pauses are better observed in hearts driven at such quick rhythms that the refractory period is made to cover a considerable part of each cycle.

A second break-shock falling at progressively increasing intervals after a previous one, begins to evoke summated contractions as soon as the refractory period is over. The recovery curve of the contractile power gave gradually rising curves, comparable to the similar curve for the frog's heart, after neutral or alkaline perfusion.

By repetition of stimuli of the same strength—

(*a*) at intervals for which relaxation is not yet complete, the heights of the contractions rise in a *true staircase* arrangement;

(*b*) at shorter intervals, new steps of summation occur, followed by undulatory or complete tetanus.

Whether the high frequency of stimulation is reached suddenly or slowly, a drop of a half-rhythm is never observed—as in the vertebrate heart—because fusion of contraction occurs earlier.

I am indebted to Dr. E. J. Allen and the Staff of the Marine Biological Laboratory at Plymouth for their interest and assistance in carrying out this work.

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On the Composite Nature of Certain Potato Virus Diseases of the Mosaic Group as Revealed by the use of Plant Indicators and Selective Methods of Transmission.

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[PLATES 30–33.]

I. *Introduction.*

In 1928 some experiments by the writer (1929) on the transmission of a potato mosaic virus to tobacco revealed the curious fact that needle and aphis (*Myzus persicæ*) inoculation respectively, from the same mosaic-affected potato plant, produced in tobacco symptoms characteristic of the mode of transmission. This phenomenon was the first definite evidence of the composite nature of potato mosaic diseases, although its exact significance was not at first realised. The following year a parallel set of experiments, carried out with the potato virus disease known as "crinkle," revealed the same phenomenon, *i.e.*, the production in tobacco of symptoms characteristic of the method of infection (*1930a*). In this case, however, although the disease produced in tobacco from the crinkle potato by the aphis *Myzus persicæ* was similar to that produced by the aphis in the first set of experiments with mosaic potato, the parallel symptoms arising in tobacco by needle inoculation from the two sources were not identical. Valleau and Johnson (1930) also showed that the American potato disease known as "rugose mosaic," probably the same disease as the above-mentioned crinkle, contained two viruses, one of which they refer to as "veinbanding." This is apparently identical with the disease produced in tobacco by the aphis in the writer's experiments. The other virus they call the "healthy potato virus," which is similar to that shown by James Johnson (1925) to be almost universally present in American potatoes.

In 1931 the writer published a short account of the results achieved in four years' study of the composite nature of potato mosaic viruses, outlining the technique of virus isolation from a complex in the living plant, which is more fully described in the present paper. Later Koch (1931) published a statement of the results of work carried on in James Johnson's laboratory on rugose mosaic of potato, which appears to confirm the writer's findings with "crinkle."

A serious hindrance to the study of plant—and especially of potato—viruses is the lack of any sound system of classification. Hitherto, potato virus workers have been compelled to classify the disease according to the symptoms produced on the potato, and this system has now proved entirely inadequate. No doubt it will be necessary for some time to continue to refer to certain potato diseases as "crinkle," "mosaic" or "streak," in order to describe a set of *symptoms*, but such designations really have no bearing on the actual viruses concerned with the production of those symptoms. The writer has found that what has been known up to the present as "streak" may sometimes consist of no less than three viruses, while the potato Up-to-Date, well known as a "streak"-carrier, almost invariably contains two viruses.

James Johnson (1927) has attempted to classify plant viruses on a basis of certain physical properties, such as longevity *in vitro*, thermal death-point, tolerance to dilution and so on. Quanjer (1931) has recently put forward a system of classification of potato viruses based on the morbid anatomy of the plant. By restricting himself, however, to one method of virus transmission, *i.e.*, grafting, and by the use of potato plants only in his studies, Quanjer failed to realise the composite nature of many of the diseases with which he was working. The potato plant has only a limited range of symptom expression, and it has not a specific reaction for each and every virus. It is necessary therefore, in order to make a comprehensive study of potato viruses, to use other Solanaceous plants which will react differently to different viruses; such plants the writer has termed *plant indicators*. Again, it is necessary to invoke the aid of a specific virus resistance exhibited by certain other plants in order to split up a virus complex; such plants are referred to here as *plant filters*. Finally, by using more than one method of transmission, it is possible to prove the existence of a virus complex, and to obtain a "pure culture" of one virus; this can be achieved, for example, by making use of the selective powers of virus transmission possessed by the aphis vector.

In the writer's opinion, therefore, it is somewhat premature to attempt to classify potato virus diseases until these diseases have been analysed into their component viruses, and until there is sufficient evidence to show that a single entity only is under consideration.

Acknowledgment is due to Mr. J. P. Doncaster for his assistance during the latter part of this work, and particularly for his help in taking the photographs.

II. *On a Technique of Virus Isolation from a Complex within the Living Plant.*

The technique used in the separation of the various mosaic viruses affecting the potato plant is based upon a number of observations made by the writer in the course of his studies upon potato virus diseases. These methods of virus isolation fall into three groups and separation was effected in the following ways:—

(1) By utilising the selective power of transmission from a virus complex exhibited by the aphis *Myzus persicæ*.

(2) By the use of plant indicators and plant filters.

(3) By taking advantage, firstly, of the difference in the lengths of the incubation periods of the constituent viruses of a complex within the tobacco plant, and secondly of their unequal rates of movement: (*a*) at the moment of the development of primary symptoms in the young plant inoculated with the virus complex, (*b*) in the ageing plant.

Before describing more fully these methods of virus isolation, it will be well to state that the virus complex dealt with in this paper consists of two viruses carried without symptoms by the potato variety Up-to-Date, and hereinafter referred to, for the sake of clarity, as x and y, where x is the needle-transmitted virus, not aphis-borne, while y is the aphis-borne virus which is also transmissible by the needle. It should be remembered, therefore, that the needle transmits the whole complex $x + y$, but the aphis transmits the virus y only.

The reactions of these two viruses upon various plant hosts are described in detail in Section III.

In describing the technique of virus isolation, the selective power of transmission exhibited by the aphis *Myzus persicæ* will be dealt with first. During the period of study, some twenty examples of potato virus diseases of the mosaic group have been subjected to comparative transmission tests to tobacco, by aphis and needle respectively. While many of these diseases proved to be due to mixtures of viruses, others consisted of the aphis-borne virus *y* alone. In no single case has the aphis *Myzus persicæ* transmitted the whole virus complex to tobacco, or any other plant, including the potato, although the complex was itself quite easily transmissible as a whole by needle scratch. In each case the insect has selected the same virus, referred to here as *y* and described in an ensuing section of this paper. It would seem, therefore, at present, as if the other potato virus, or viruses, of the mosaic group are not aphis-borne. Incidentally, it may be mentioned that when the aphis *Myzus persicæ* is colonised upon a potato plant containing a complex consisting of leaf roll, the *y* virus and the *x* virus, the first two only are transmitted by the insect. Such selective powers of transmission by this and related aphides have been recorded in respect of other plant viruses. Bennett (1930) has shown that two species of aphides, *Amphorophora rubi* and *Aphis rubiphila*, exhibit a selective preference in regard to raspberry viruses, while Hoggan (1929) has shown that *M. persicæ* will select a cucumber mosaic virus from a combination of this and tobacco mosaic upon a tobacco plant.

By making use, therefore, of this curious selective preference on the part of an insect vector, the investigator is provided with a ready means of securing a " pure culture " of one particular virus.

Turning now to the consideration of the second method of virus isolation; this involves the use of particular plants which may be employed as " indicators " or " filters." Having achieved by means of the insect a method of extracting a single virus, it is necessary to use a plant host which will indicate by a precise and constant reaction the nature of the virus thus isolated. The potato plant itself is useless, owing to the wide differences in varietal reaction, and to the ever-present danger of concealed viruses with the resulting complications due to their presence. A plant which does fulfil this purpose, however, is the tobacco, two varieties of which have been extensively used by the writer—*i.e.*, White Burley and Virginia—the first-named being the most sensitive to infection by the insect. Other plants used in this connection are the tomato, *Hyoscyamus niger*, and *Solanum nigrum*.

Now, as regards plant filters, a comparative study of the host range of the virus y, isolated by means of the insect, and of the complex $x + y$ produced by needle inoculation to tobacco, revealed the fact that while the majority of the plants were susceptible to the virus x, some plants appeared to be resistant to the aphis-borne virus y. This fact suggested the possibility of using certain of these y-resistant plants as filters, and experiment has shown that passage of the whole complex $x + y$ through such plants removes the aphis-borne virus y. That the virus y is actually removed by this process is shown by the analysis and synthesis of the complex frequently carried out, and by the fact that the aphis cannot pick up the virus y from a disease produced in tobacco after passage of the complex through one of these filter plants. Examples of plants susceptible to the virus y, isolated by means of the aphis, are *Hyoscyamus niger*, tomato, and *S. nigrum*, and of the filter plants resistant to this virus, *Datura stramonium* and *Solanum dulcamara*. All are susceptible to the virus x. Having thus found the y-resistant and x-susceptible plants which could be used to filter out the aphis-borne virus and to give a " pure culture " of x, it remained to discover a plant which would filter out the other component virus—in other words, an x-resistant and y-susceptible plant. This could be used as an alternative method for isolating y in place of the aphis. After some search, such a plant was found, viz., *Petunia*.* More than fifty inoculations of the $x + y$ complex through young *Petunia* plants back to tobacco have yielded a " pure culture " of y (Plate 31, fig. 6), while attempts to infect *Petunia* with a " pure culture " of x by needle-scratch have consistently failed.

It should perhaps be pointed out that the successful use of these filter plants depends upon the needle-scratch method of inoculation; grafting appears to transmit the whole complex, at all events in some cases.

The third method of virus isolation is based on an interesting difference in the rate of movement of the respective viruses in the tobacco plant. When a young tobacco plant of the varieties White Burley or Virginia is inoculated by needle-scratch with the virus complex $x + y$, the first symptoms develop four days later under the writer's conditions (maximum temperature 80° F., minimum 50° F.). These initial symptoms take the form of double concentric rings, usually with a central spot, clustered on and around the needle scratches (Plate 31, fig. 7); this is the virus x. Three days later, giving a total incubation period of 7 days, the initial symptoms of the virus y appear; these take the invariable form of a " clearing of the veins " of the youngest leaf (Plate 31, fig. 8). The next development is the appearance of necrotic lesions in the young leaves showing this clearing of the veins, indicating that the virus x has also reached the young leaves, but as the virus y is already present there in an active form, the rings characteristic of x alone are not formed. This takes place about 2 days after the first appearance of clearing of the veins. The two viruses then combine to give the virulent necrotic disease which is illustrated in Plate 30, fig. 2. It appears, then, from a study of this symptom sequence that the virus x has the short incubation period of 4 days, and appears first; it is thus able to show in its ring-like form upon the inoculated leaf as if it were a single entity. On the other hand, although the virus y has the longer incubation period of 7 days, it moves more rapidly through the plant, and appears in the youngest leaves some two days before the more slowly-moving virus x. It is easy, therefore, to obtain a " pure culture " of the virus y by sub-inoculating from the young leaves on the first appearance of the clearing of the veins. As regards the isolation of the virus x by this method, the position is less certain. If sub-inoculations are made from the inoculated leaf immediately on the appearance of the double rings, the whole complex $x + y$ is brought over, showing that the virus y is present, although as yet showing no symptoms. On the other hand, the writer has sometimes been able to obtain a " pure culture " of x by waiting until the clearing of the veins has developed in the young leaves before sub-inoculating from the inoculated leaf. It appears, therefore, as if there may be a short period when the two viruses are completely separated and exist independently in the plant. At the same time, although it is clear that the y virus, by its more rapid movement, is separated for a time from the x virus, there still remains some doubt as to whether, in an $x + y$ complex, the x virus ever becomes completely divorced from the accompanying y. A somewhat similar phenomenon is shown in the maturing tobacco plant affected with the $x + y$ complex: the y virus may sometimes be found temporarily alone in the topmost leaves, while the lower leaves contain both viruses. Valleau and Johnson (1930) have also found it possible to effect a separation by inoculating from the etiolated sprouts of potatoes grown in a dark cellar, as compared with transfers from halves of the same tubers grown in the greenhouse.

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* Garden variety.

The reasons for this different behaviour of the viruses x and y in the tobacco plant are not very clear. It may possibly be that the two viruses do not move in the same channels in the plant tissue, or there may be a difference in size of the particles of the respective viruses which might account for the different rates of movement. Whether there is any connection between a possible difference of location in the plant tissues of the two viruses and the inability of the aphis to transmit the x virus, remains to be proved. Further research is being carried on into the question of the movement of these two viruses in the plant.

It may be of interest to describe an actual analysis and synthesis of the $x + y$ complex in the tobacco plant as frequently carried out, using some of the analytical methods above described. So far as the writer is aware, this is the first time a virus complex has been analysed and synthesised in the living plant. The experiment was performed as follows, the source of infection being a streak-carrying Up-to-Date potato plant which exhibited no symptoms. Needle inoculation was made from this plant to White Burley tobacco seedlings; this resulted in the production of a virulent disease characterised by gross lesions and severe necrosis of the veins (Plate 30, fig. 2). A parallel series of infection experiments was carried out from the same Up-to-Date potato plant to White Burley by means of the aphis *Myzus persicæ*; this resulted in the production of the disease y with the characteristic darkening of the green colour of the tissues along the veins (Plate 30, fig. 1). On passage of the necrotic complex through one of the filter plants referred to, back to tobacco again, the disease took on a "ringspot" form, with numbers of clearly-defined double concentric rings, but there was no general necrosis. This is a typical picture of the virus x (Plate 30, fig. 3). Now to a number of these tobacco plants showing the ringspot symptoms of x was added the virus y, which had been isolated by means of the aphis. After the usual incubation period, the primary symptoms peculiar to the aphis-transmitted virus y appeared; following rapidly upon this, the rings lost their regular outline, became filled up, and degenerated into irregular necrotic lesions, while a severe necrosis of the veins developed. In a short time the symptom picture was identical with that of the necrotic disease before the separation had been effected (Plate 30, fig. 4). Further passage through the filter plants again produced the ringspot disease x, while the addition to this of the aphis-carried virus y once more restored the virulent necrotic picture. The same analysis and synthesis can, of course, be carried out by substituting the *Petunia* plant for the *Datura*. The x virus is thus removed from the $x + y$ complex instead of y, but if the x virus is added to the y which remains after passage of the complex through *Petunia*, the complex is synthesised once more.

III. *A Study of the Reactions of the Isolated Viruses,* x *and* y, *upon a Selected Range of Host Plants.*

A study of the symptom expression of the x and y viruses upon a selected range of Solanaceous plants has been carried out, and the results are given herewith. It is hoped later to develop a selection of plant species whose reactions may become a standard test for the identification and classification of potato mosaic viruses. The plants used in the present work are as follows :—

(1) *Nicotiana tabacum*, Tobacco, White Burley and Virginia.
(2) *Datura stramonium.*
(3) *Solanum dulcamara.*
(4) *Solanum nigrum.*
(5) Tomato.
(6) *Hyoscyamus niger.*
(7) *Petunia.*
(8) Potato—
    (*a*) President.
    (*b*) Arran Victory.
    (*c*) " Majestic " seedlings.

The reactions of the two viruses upon these plants will be treated separately, the x virus being dealt with first. It is perhaps important to state that, in every example of the following series of x and y infections, return inoculations have been made to White Burley tobacco, and in every case a " pure culture " of the x or y virus was once more obtained, except, of course, in those cases where the plant was x- or y-resistant. This is important confirmatory evidence that the symptoms obtained in the various plant species were actually due to the x or the y viruses, and to no other cause.

A. *Reactions of the* x *Virus.*

(1) *Nicotiana tabacum* (Tobacco), White Burley and Virginia varieties.—As already stated in Section II, the first symptoms of the x virus on tobacco take the form of double concentric rings or occasionally single rings on the inoculation scratches. These develop with fair constancy 4 days after inoculation, when kept at an average daily temperature of 70° F. After a further period of about 5 days or sometimes longer, the secondary symptoms develop, double rings appearing in groups on the other leaves (Plate 30, fig. 3). The symptoms

differ slightly in the two varieties of tobacco used, the rings being more sharply defined in Virginia than in White Burley. The size of the rings, however, is not constant. As the plant grows the rings lose their individuality somewhat, and a certain amount of necrosis of the veins may develop, particularly in White Burley, which reacts more severely to the virus than does Virginia. In an old tobacco plant affected with the x virus, the rings have usually disappeared, and the symptoms appear as a vague mosaic mottling, with some banding of the veins. Inoculation, however, from such a plant to young tobacco seedlings produces once more the typical ring disease. It is not quite clear whether this virus is identical with the American potato virus, first described by James Johnson (1925), and also called ringspot by him. The writer considers that the two are probably the same, for although the rings as portrayed by Johnson are not concentric and are often incomplete, yet it has been found possible to increase and decrease the virulence of the x virus and, in so doing, to alter its symptom expression.

(2) *Datura stramonium.*—This plant is very susceptible to the x virus, and sometimes reacts with great violence. The most severe reaction takes the form of gross lesions, while occasionally the whole leaf is killed by a severe necrosis of the " scorch " type (Plate 33, fig. 13). The more usual reaction is the development of small double rings on the middle leaves ; these rings rapidly disappear with continued growth, and their place is taken by a mosaic mottle of dark and light green or yellow, often accompanied by a darkening of the green colour especially near the veins. The primary symptoms always take the form of small dark necrotic lesions along the needle scratches ; these lesions are circular in form, but differ as a rule from the actual rings which may develop later. There is evidence that the x virus is susceptible to variations in virulence (Smith, 1929*b*), and constant successive inoculations through *Datura* tend to bring about an attenuation. The incubation periods for the x virus in *Datura* are about the same as in the tobacco plant.

(3) *Solanum dulcamara.*—*S. dulcamara* is readily infected with the x virus by needle inoculation. The disease develops in about 10 days, with the usual primary symptoms of dark circular lesions—not rings usually—on the needle scratches. Later quite clearly-defined rings with a central spot develop on the other leaves (Plate 32, fig. 12). Schaffnit is incorrect in assuming (1931) that *S. dulcamara* is immune to potato viruses. As shown in this paper, the plant is resistant to infection with the y virus by needle scratch, but is quite easily infected with the x virus by the same method of transmission. As *S. dulcamara* grows, the symptoms tend to disappear, and the plant is capable of acting as a " carrier " of the virus.

(4) *Solanum nigrum.*—*S. nigrum* is easily infected with the x virus, and symptoms develop in about 8 days. The general appearance of the disease is similar to that produced in *S. dulcamara*, although the reaction is usually more severe; concentric rings develop on this plant also. It is worth noting that *S. nigrum*, which is a common weed in the potato fields round Cambridge, is never found affected with the x virus in nature; this is a very different state of affairs from that which exists in the case of *S. nigrum* and the y virus (p. 262).

(5) *Tomato.*—As in the other plants, the x virus produces dark circular lesions on the inoculated leaf from 5 to 7 days after inoculation; concurrently with these local lesions, some small necrotic spots may develop on other leaves. A few days later large numbers of necrotic lesions of a ring-like form appear (Plate 32, fig. 10); these increase in severity (Plate 32, fig. 11), and finally a streak disease of the typical leaf-drop type develops. Occasionally fairly clear double concentric rings with necrotic margins also occur. Although the tomato plant is affected in nature with several diseases of the " streak " type, it is unlikely, in the writer's opinion, that the x virus is one of them, as it is apparently not aphis-borne.

(6) *Hyoscyamus niger.*—This plant is very susceptible to the x virus, and may be killed by it. Primary symptoms are the development of large lesions on the inoculated leaf, followed a few days later by ring-like lesions on the other leaves (Plate 33, fig. 16).

(7) *Petunia.*—*Petunia* is apparently resistant to infection by needle-scratch with the x virus, and is the x-resistant and y-susceptible plant used in separating the complex. Repeated needle inoculations with the $x + y$ complex have been made to *Petunia* plants, but in every case the *Petunia* has developed only the symptoms of the y virus (Plate 31, fig. 5); return inoculation from such *Petunia* plants to tobacco have produced only the y virus (Plate 31, fig. 6). Inoculations with a " pure culture " of the x virus to *Petunia* have produced no results.

(8) *Potato.*—(*a*) *President.* Primary symptoms in the form of dark red necrotic lesions develop along the needle-scratches after about 8 to 10 days. About 10 days later, giving a total incubation period of approximately 21 days, a mosaic mottling develops on the topmost leaves, sometimes accompanied by a crinkling and distortion of the leaf edges. There are seldom any lesions of the " streak " type in this variety with the x virus.

(*b*) *Arran Victory.* The same type of necrotic lesions develop on the inoculated leaf of this variety also (Plate 32, fig. 9). These are followed about 14 days later by a mosaic mottling on the topmost leaves. The mottling is well marked, exhibiting numbers of fairly bright yellow spots intermingled with light or dark green. Arran Victory often gives a "streak" reaction to the *x* virus in the current season. This takes the form of irregular lesions on the lower leaves, which gradually spread; the leaves then turn yellow and drop.

(*c*) "*Majestic*" *Seedlings.*—On the whole the reactions to the *x* virus of a number of seedlings, derived from Majestic potato, resembled the symptoms produced on President by the same virus. Several of the seedlings showed a well-marked mosaic mottlng without any lesions, while in some cases irregular necrotic lesions developed on the lower leaves.

B. *Reactions of the* y *Virus.*

The symptom sequence and expression of the *y* virus is remarkably constant upon a variety of plant hosts, with the exception of the potato plant, as is shown by the series here described. Strains of this virus have been kept going by progressive needle inoculation through various plant hosts for 4 years, without any change in symptom expression or apparent alteration in virulence.

(1) *Nicotiana tabacum* (Tobacco), White Burley and Virginia varieties.—Primary symptoms appear after 7 days at a mean daily temperature of 70° F., and invariably take the form of "clearing of the veins" of the youngest leaf. The veins stand out in a characteristic manner; in addition, there is sometimes a slight crinkling of the leaf surface and margins. This clearing of the veins soon disappears, and its place is taken some days later by a darkening of the green colour in the tissues along the veins (Plate 31, fig. 6). There is little difference in reaction to the *y* virus between the two tobacco varieties, White Burley and Virginia, when needle-inoculated, but for some reason the Virginia variety appears to be more difficult to infect with the virus by means of the aphis *Myzus persicæ*. That the *y* virus, as isolated by the writer from many different sources, is apparently the same entity is shown by the following experiment. The virus obtained from six different examples of potato virus diseases was inoculated to healthy White Burley seedlings in combinations of 2, 3, 4, 5 and 6 *y* viruses; in every case the disease produced was the normal one, and differed in no degree from the usual expression of the *y* virus.

(2) *Datura stramonium.*—This plant is definitely resistant to infection with the *y* virus by needle-scratch. Out of many attempts to infect *Datura* with this virus, carried out over 4 years, not one has been successful. On occasion. however, it appears to have been transmitted by grafting.

(3) *Solanum dulcamara.*—Like *Datura stramonium*, this plant is apparently

resistant to the y virus by needle-scratch, and it has been used in conjunction with *Datura* as a filter plant for separating the $x + y$ complex. Grafting appears to transmit the virus to *S. dulcamara*, but it is carried without symptoms, and on grafting back from such a carrier to healthy President potato, the symptoms typical of the y virus developed in it.

(4) *Solanum nigrum.*—Unlike *Solanum dulcamara*, *S. nigrum* is susceptible to the y virus by both needle and aphis (*Myzus persicæ*) agency. Clearing of the veins is hardly discernible in *S. nigrum*, but the characteristic darkening of the green colour along the veins is well shown. After a short time these symptoms disappear, and the plant becomes a carrier, a fact already pointed out by the writer (1930*b*). *Solanum nigrum* affected with the y virus occurs commonly in the potato fields in the vicinity of Cambridge.

(5) *Tomato.*—The tomato plant is readily susceptible to infection with the y virus by both needle and aphis agency. The symptom expression follows the usual sequence, but unless carefully watched for may be easily missed. Clearing of the veins develops after 7 to 9 days, followed a few days later by the characteristic darkening of the green colour of the tissues along the veins. The symptoms are never strongly marked, but are clear enough to the trained observer; moreover, confirmatory proof was always forthcoming by return inoculation to tobacco. As the plant grows the symptoms entirely disappear, and the tomato " carries " the virus without symptoms. It is probable that the tomato plant is much more often infected with this virus than is commonly supposed, and in districts or countries where tomatoes are grown out of doors, especially if near potatoes, the percentage of infection is likely to be very high. In this connection the following observation is of interest: a number of tomato seedlings were found growing by chance in a field in close proximity to a potato crop, and removed to the insect-proof glasshouse for observation; in a short time several plants developed typical symptoms of infection with the y virus which had obviously been transmitted through the agency of the aphis *M. persicæ* from the neighbouring potato plants.

(6) *Hyoscyamus niger.*—The symptoms of y infection upon *Hyoscyamus* resemble very closely those produced on tobacco by the same virus; clearing of the veins develops in 7 days, followed a few days later by the characteristic darkening along the veins (Plate 33, fig. 15). There is no necrosis formed, as is the case with the x virus (*cf.* Plate 33, fig. 16). *Hyoscyamus* is easily infected by both aphis and needle, and has been used fairly extensively by the writer as an indicator plant.

(7) *Petunia.*—As has been shown in Section II, *Petunia* is susceptible to the

y virus although resistant to *x*. Clearing of the veins is hardly perceptible in this plant, but the darkening of the green colour along the veins is quite distinct (Plate 31, fig. 5).

.(8) *Potato.*—(*a*) *President.* A description has already been given by the writer (1930*a*) of the symptoms of the *y* virus on President potato, and they are only briefly recapitulated here. Current season symptoms take the form of longitudinal streaks running along the veins on the underside of the leaf (Plate 33, fig. 14). These are followed by a severe streak of the leaf-drop type (1930*a*); the top of the plant remains unaffected by streak symptoms, but develops a marked crinkle. The following year there is little or no necrosis, but the disease takes the form of a crinkle, while occasionally it occurs in the rosette form known as "curly dwarf."

(*b*) *Arran Victory.* The *y* virus produces symptoms of mosaic mottling on this variety, sometimes accompanied by a certain amount of crinkling and leaf distortion; there is no leaf-drop streak, though occasionally small necrotic spots may develop. The writer has observed Arran Victory plants in the field affected with the browning of the leaves, sometimes known as "rust." Such plants have always yielded the *y* virus, although the connection between this virus and "rust" cannot yet be said to be definitely established.

(*c*) *Majestic Seedlings.* A large number of seedlings derived from Majestic potato have been inoculated with the *y* virus. The reaction of the seedlings has been remarkably constant, considering their differing character. The disease produced resembled that in President, but was usually rather more severe, the streak lesions were larger and more blotchy in shape, and the leaf-drop streak which followed occasionally killed the plant. Usually the top leaves remained unaffected by streak, but developed a mosaic mottling.

IV. *Discussion.*

In considering the relationship of the *x* and *y* viruses to potato mosaic diseases, it is important to realise the different nature of the two viruses concerned. While the aphis-borne virus *y* appears to be a constant entity not liable to fluctuations in virulence, the virus *x*, on the other hand, is susceptible to considerable changes in virulence, and exhibits itself in several symptom expressions, which may take the form of a faint mottle, a type of "veinbanding" or rings of several kinds.

There are two factors, therefore, which have given rise to the confusion which exists at the present time with regard to potato mosaic diseases. The first is the dual nature, hitherto unsuspected, of so many of the potato virus diseases of the mosaic group, and the second is the fluctuation in virulence exhibited by one constituent, *i.e.*, x, of these diseases. In the writer's opinion, the different symptoms exhibited by the various potato mosaic diseases are due rather to strains of the x virus, differing in virulence, than to distinct viruses. Thus it will probably be found that these two viruses, x and y, alone or in combination, are responsible for the majority, though not all, of potato mosaic diseases.

There is little doubt of the almost universal occurrence of the aphis-borne virus y in potato virus diseases. In this respect it rivals the American "healthy potato virus," except that the y virus is usually associated with symptoms of the crinkle type, although the writer has twice isolated it from the symptomless carriers, Up-to-Date and Di Vernon. So far the y virus has been obtained from twenty examples of the potato diseases—mosaic, crinkle and streak. In the field it has also been found, apparently alone, causing a crinkle or mosaic in President, Arran Victory and Epicure, while alone or in combination with the other virus it causes current season symptoms of leaf-drop streak in Up-to-Date. Owing to its infectious nature and affinity with the aphis *Myzus persicæ*, this wide distribution of the y virus is not surprising, nor is this occurrence confined to the potato plant alone. It has been shown to occur commonly in the weed *Solanum nigrum* (Smith, 1930*b*) and in the tomato, and presumably where *Hyoscyamus niger* occurs in the field that plant would be infected also; that this virus is common in the tobacco fields of Kentucky has been shown by Valleau (1930). The distribution of the x virus is also very wide, and in varying degrees of virulence it has been found on many occasions in nature. Further research is needed, and is being carried out on the question of the insect vectors of this virus; at the moment, as shown in this paper, it appears as if the x virus is not aphis-borne, as all attempts to induce a number of aphis species to transmit it have so far failed.

As regards the "plant indicators," it is perhaps too much to expect that one plant species, or even two, will be sufficient to give a precise reaction for each separate potato virus, and it will probably be necessary to look for other plant indicators. It is true that the tobacco plant is a sensitive indicator of x and y, while *Datura* seems to react readily to x in all stages of virulence. Nevertheless, it is quite possible that there may exist one or more potato viruses which give no reaction on these two plants. Further research is necessary on this point.

V. *Summary.*

(1) Evidence is presented which proves that many of the potato virus diseases, known as mosaic, crinkle and streak, are composite in nature.

(2) A technique of virus isolation from a complex in the living plant is described. The methods of virus isolation fall into three groups, and separation was effected in the following ways :—

(*a*) By utilising the selective power of virus transmission exhibited by the aphis *Myzus persicæ* Sulz.

(*b*) By the use of *plant indicators* and *plant filters*. The first type of plant shows by its specific reactions when more than one virus is concerned, while passage of the complex through the second type of plant removes one or other of the component viruses of the complex to which the filter plant may be resistant. An example of a plant indicator is *Nicotiana tabacum*, while *Datura stramonium*, *Solanum dulcamara* and *Petunia* are examples of plant filters.

(*c*) By taking advantage, firstly, of the difference in the lengths of the incubation periods, and, secondly, of the unequal rates of movement of the constituent viruses of a complex within the tobacco plant, at the moment of the development of primary symptoms in the young plant inoculated with the virus complex ; or in the ageing plant.

(3) Two viruses, x and y, isolated from a symptomless "streak"-carrier potato, Up-to-Date, are described, and their reactions upon a selected range of host plants are given. The virus x is transmissible by needle-scratch, but not by the agency of aphides ; the virus y is transmissible by both methods. The virus y has been shown to be an almost invariable constituent of potato virus diseases of the mosaic group ; it has been isolated from twenty examples of potato diseases of this group, and has also been found alone, causing "crinkle" in several potato varieties.

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EXPLANATION OF PLATES.

PLATE 30.

The photographs in this plate illustrate the analysis and synthesis of a virus complex.

FIG. 1.—Leaf of a White Burley tobacco plant infected by means of the aphis *Myzus persicæ* Sulz. from a streak-carrying Up-to-Date potato plant. This is the aphis-borne virus *y*.

FIG. 2.—White Burley tobacco infected by means of the needle from the same Up-to-Date potato. Note the severe necrosis and general virulence of the disease. This is the virus complex $x + y$.

FIG. 3.—Leaf of White Burley tobacco infected by means of the needle from the plant shown in fig. 2, after passage of a *y*-resistant filter plant. This is the "ringspot" virus *x*, separated from the accompanying virus *y*.

FIG. 4.—Leaf of the same White Burley plant shown in fig. 3, to which the *y* virus has been again returned; the necrotic symptom picture typical of the virus complex, as shown in fig. 2, is restored.

PLATE 31.

FIG. 5.—Leaf of *Petunia*, an *x*-resistant plant, inoculated by the needle with the $x + y$ virus complex from the tobacco plant shown in fig. 2. Note that the *x* virus has been filtered out, and only the *y* virus has developed, characterised by the usual dark green colour along the veins.

FIG. 6.—Leaf of White Burley tobacco plant inoculated from the *Petunia* shown in fig. 5. Only the *y* virus has developed, showing that the *x* virus has actually been filtered out by the *Petunia*.

FIG. 7.—Primary symptoms of the *x* virus on tobacco; rings develop on the inoculated leaf 4 days after inoculation.

FIG. 8.—Primary symptoms of the *y* virus on tobacco: "clearing of the veins" of the young leaves develops 7 days after infection.

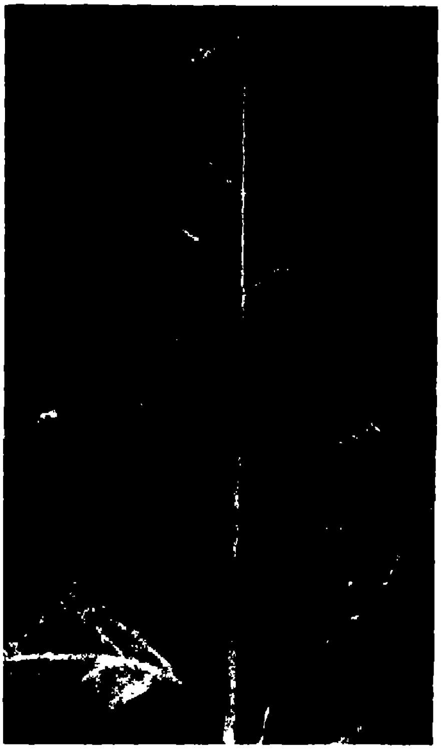
PLATE 32.

FIG. 9.—Primary symptoms of the *x* virus on potato Arran Victory. Local necrotic lesions on the inoculated leaf are characteristic of this virus.

FIG. 10.—Symptoms of the *x* virus on tomato. Note the ring-like lesions on the leaves; primary symptoms are similar to those shown in fig. 9 for potato.

FIG. 11.—Later stage in the development of the *x* virus on tomato. These lesions are followed by the total collapse of the leaves.

FIG. 12.—Symptoms of the *x* virus upon *Solanum dulcamara*, a *y*-resistant plant. This plant was inoculated with the $x + y$ complex. Note that symptoms of *x* alone have developed.



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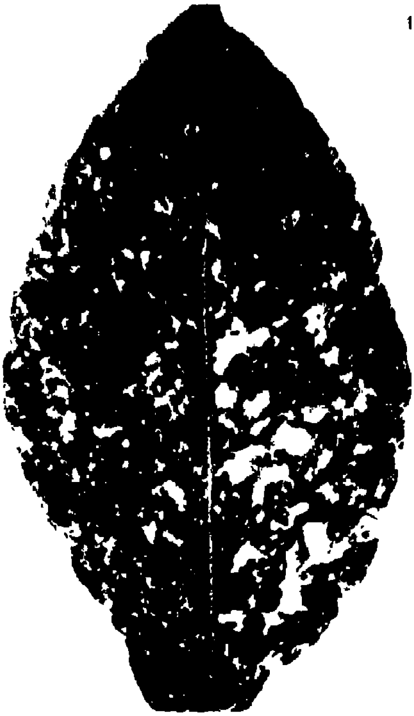

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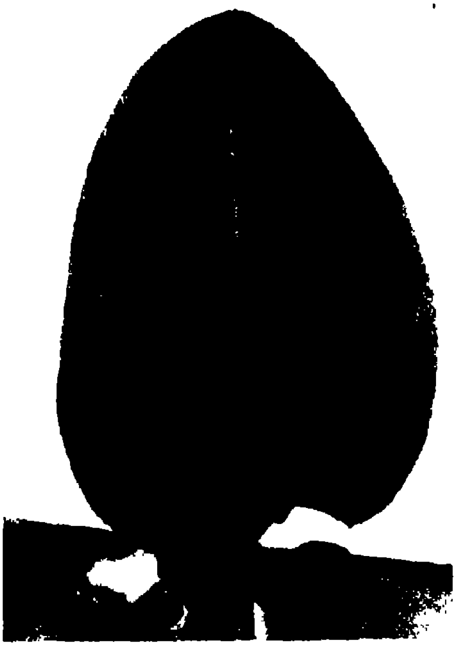

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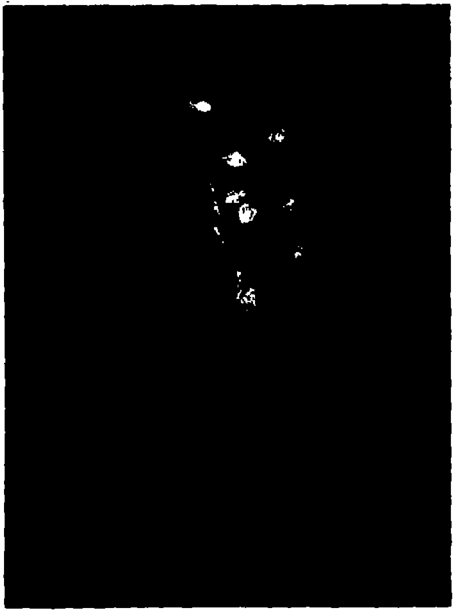

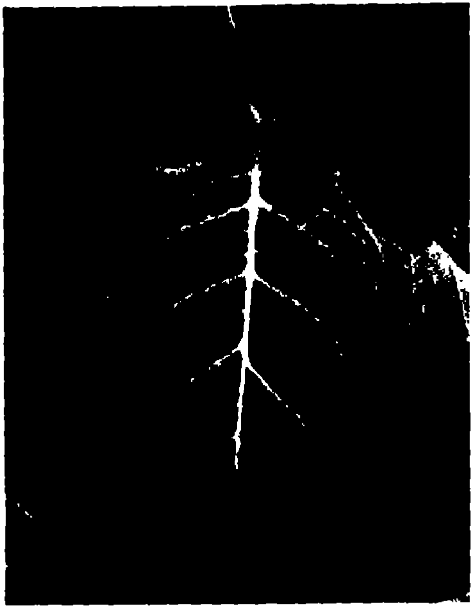

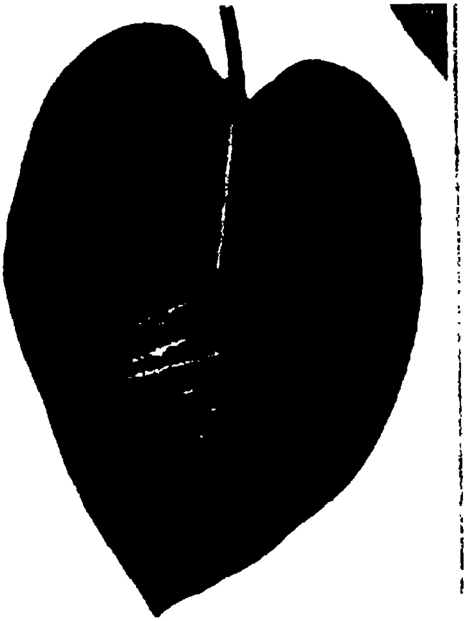

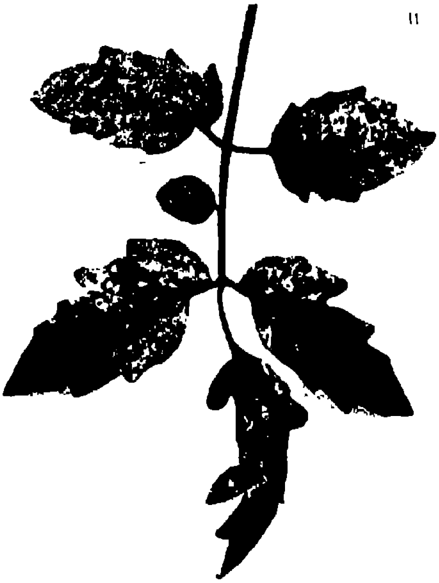

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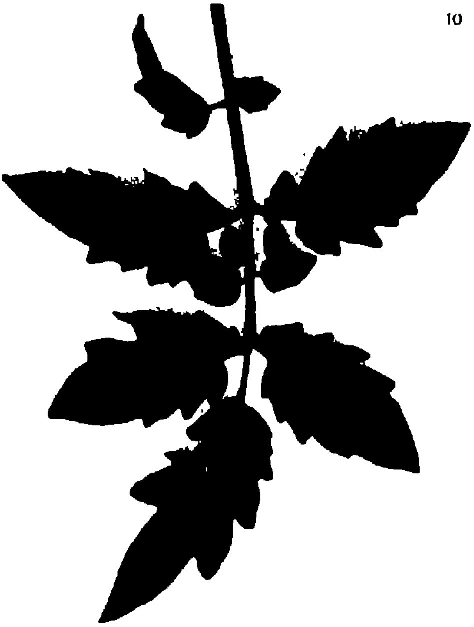

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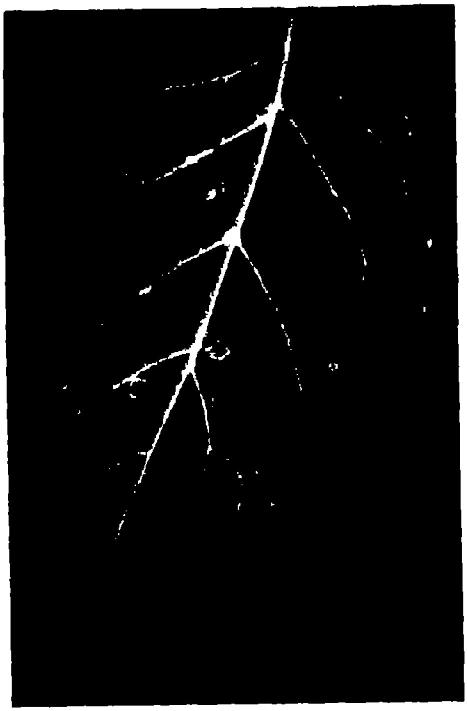

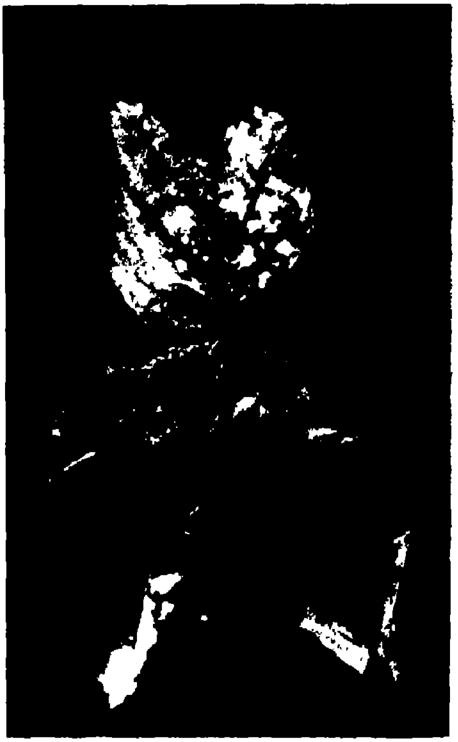

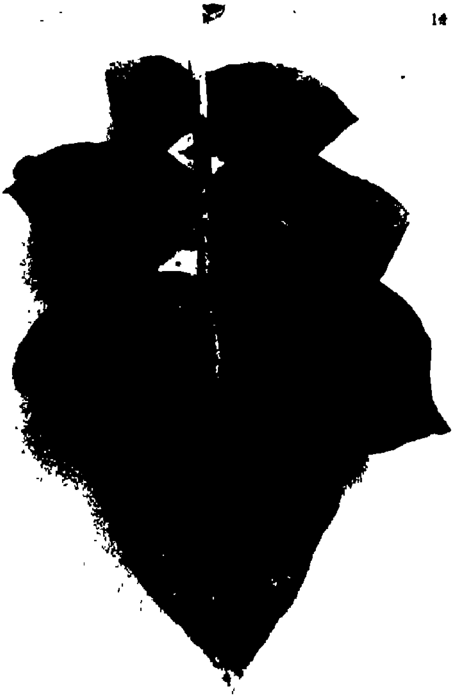

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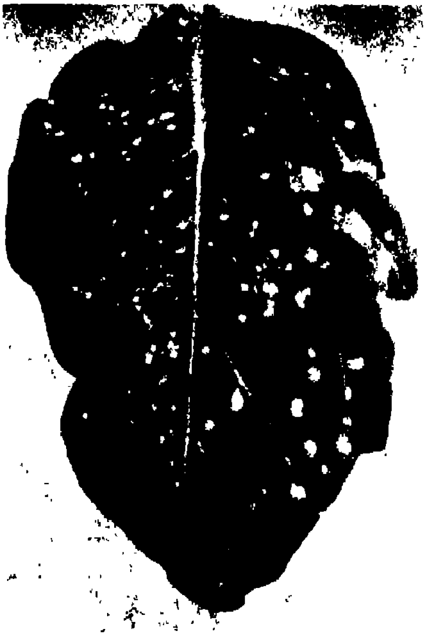

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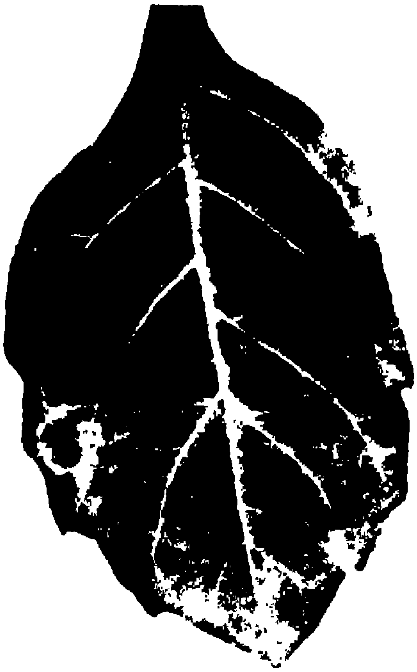

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PLATE 33.

FIG. 13.—Symptoms of the x virus upon *Datura*. In this case the disease caused was very severe.

FIG. 14.—Primary symptoms of the y virus upon potato President. Note the streaks along the veins on the underside of the leaf. These symptoms are followed by a leaf-drop streak of the lower leaves, while the top leaves develop a crinkle.

FIG. 15.—Symptoms of the y virus upon *Hyoscyamus*. Note the characteristic darkening of the green colour.

FIG. 16.—Symptoms of the x virus upon *Hyoscyamus*. Note the ring-like necrotic lesions.

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Myothermic Experiments on the Frog's Gastrocnemius.

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The gastrocnemius-sciatic preparation of the frog has been one of the chief objects on which the properties of isolated muscle have been studied. Its many obvious advantages, however, do not make it suitable for all purposes; for some it has certain important disadvantages:—

(*a*) Its size and thickness (in any but the smallest animals) render the supply of oxygen for recovery after stimulation, or even for resting metabolism, difficult or impossible. The critical diameter of a resting muscle just fully supplied with oxygen (at 20° C. in oxygen) is only 5·7 mm., and many gastrocnemii are larger than this (see Hill, 1928, *c*, pp. 60, 62). In a thick muscle the diffusion of oxygen cannot replace its normal supply by the circulation;

(*b*) The complex arrangement of its fibres and its inextensibility due to connective tissue make it unsatisfactory for studying the mechanical properties of muscle; and

(*c*) Its shape makes the measurement of its heat production by the usual myothermic methods inaccurate.

Of these disadvantages the first (*a*) might be avoided by working under a higher pressure of oxygen. Special methods, however, would be necessary, and it is not certain that the high oxygen pressure would not affect the outer layers fully exposed to it. The pressure indeed would need to be rather high, since the critical thickness of a muscle fully supplied with oxygen under any given conditions varies only as the square root of the partial pressure at its surface; for example, if a muscle 1 mm. thick requires 1 atmosphere of oxygen to maintain a steady state during activity, another muscle 7 mm. thick during similar activity would need 49 atmospheres. These numbers correspond about to sartorius and gastrocnemius respectively; if gastrocnemii only had been available little would be known about the recovery process, or about the steady state of muscles maintained in continuous activity (see Hill and Kupalov, 1929). Moreover, apart from the study of the recovery process itself, recovery is necessary in order to maintain a muscle in good condition during the more or less intense activity often required in a prolonged experiment. Gastrocnemii, therefore, unless extremely small, are suitable only for acute experiments not involving recovery.

The second disadvantage (*b*) cannot be altogether avoided. It will be shown below that, in respect of the relation between tension developed and heat produced under isometric conditions, the gastrocnemius behaves like a sartorius (the nearest approach to a parallel fibred muscle) of half its length. When allowed to shorten, however, its behaviour may not be so simple (its fibres may pull too obliquely) and moreover its inextensibility prevents us from examining the important consequences of initial stretch. For the latter reason the Fenn effect is obscured (see Rothschild, 1930; Hill, 1930, *b*). If we wish our experimental object in its behaviour directly to represent the muscle fibre, it is necessary to use a muscle more nearly consisting of parallel fibres, with as little connective tissue as possible.

For many experiments, however, and particularly for those on the anaerobic chemical changes associated with stimulation, the gastrocnemius is particularly well suited and has been largely employed. The ease, moreover, with which it (and its nerve) can be prepared without injury is a great advantage. For such reasons many attempts have been made to use it for myothermic experiments. The chief difficulty (apart from its shape, which makes contact with a thermopile rather uncertain, and its thickness, which makes heat conduction very slow) lies in the determination of the heat in absolute units. For this purpose, with any of the ordinary methods, it is necessary to "calibrate," *i.e.*, to kill the muscle (*e.g.*, by chloroform) without changing its position, to heat it with a known amount of electrical energy, and to observe the resulting deflection. In the case, however, of the gastrocnemius, the cross-section is so variable that the heating is extremely non-uniform, and the deflection depends upon the position of the thermopile on the muscle. With a given current running down the muscle between electrodes at its ends the rise of temperature at any point depends upon the *square* of the cross-section there, and since the cross-section varies very considerably the rise of temperature varies many fold. I made several attempts recently, by employing as electrodes wires wound round the muscle, to "calibrate" the middle approximately cylindrical portion of it. The irregular and inconsistent results obtained, however, left little hope of accuracy in this direction; an entirely new method, therefore, was attempted, ultimately with success.

It might have seemed possible to determine the absolute sensitivity, in °C. per millimetre deflection, by calculation from (*a*) the number of thermocouples in the thermopile, (*b*) the thermo-e.m.f. per 1° C. of the metals employed, and (*c*) the sensitivity of the galvanometer. This method has, in fact, been frequently used, but in cases where a direct calibration is made for comparison, a considerable error is found. The actual sensitivity may be only 25 to 75 per cent. of that calculated, so that observations, for example, of Tl/H (T = tension developed, l = muscle length, H = heat produced) are altogether wrong. The reasons are important, since they allow us to develop a method free from error; they are:—

(i) The heat capacity of the thermopile is not negligible in comparison with that of the muscle;

(ii) Heat loss from the thermopile, either along the wires or into the air around, prevents the "hot" junctions from warming to the same extent as the muscle; and

(iii) Unless the cold junctions be very far away, or heavily "loaded," their temperature rises by conduction along the wires, so that the difference of temperature observed is too small.

All these factors can be eliminated by using a single thermocouple of very fine wire stuck into and drawn through the muscle substance, and by having the cold junctions far away and of rather large dimensions. The second condition is as important as the first.

The use of a single thermocouple for measuring muscle heat is not new. Becquerel and Breschet (1835) used couples of platinum-iron or copper-iron, of 0·5 mm. diameter, the value of 1 scale division being 0·1° C., reading being to 0·01° C. Helmholtz (1847) used thermopiles consisting of 3 to 6 needles stuck into the muscles, readings being to 0·001° C. Meyerstein and Thiry

(1863) used a small thermopile of 2 needles (german silver-iron), 1 scale division being 0·0024° C. Blix (1901) made a perfectly workable apparatus, which I have used myself (Hill, 1910), in which a single copper-constantan couple was connected to the low resistance copper coil of a Broca galvanometer ; in this, however, the thermo-junction was upon the surface of, and not inside the muscle. No further attempts have, to my knowledge, been made in recent years.

The couples employed in the present research were of constantan-iron, 120 μ in diameter, giving a thermo-e.m.f. of 54·3 microvolts per 1° C. Different samples of the metals give different values, so the same sample was used throughout. The thermo-e.m.f. of each metal against copper was determined by a potentiometer and a standard thermometer, over a range of about 20° ; copper-iron gave 11·23, 11·19, 11·25, mean 11·22 microvolts per 1° C. ; copper-constantan gave 43·05, 43·10, mean 43·08 microvolts per 1° C. By addition we obtain 54·3 for the thermo-e.m.f. of constantan-iron. In the length employed each couple had a resistance of about 2 ohms. A galvanometer of the astatic moving magnet type, constructed by Mr. A. C. Downing, was used. It was nearly critically damped. Its resistance with four 12-ohm coils in parallel was 3 ohms. Thus 1° C., supplying $54 \cdot 3 \times 10^{-6}$ volt, gives $10 \cdot 86 \times 10^{-6}$ amp. The initial heat production in a single muscle twitch causes a rise of temperature of about 0·003° C. which produces $3 \cdot 26 \times 10^{-8}$ amp. The galvanometer can readily be adjusted to give 50 mm. deflection for this current, so that sufficient sensitivity is available.

The wires of the thermocouple were silver-soldered end to end, no trace of the junction being visible. It needs some skill and care to make a couple of this kind, and I am greatly indebted to Mr. A. C. Downing for his patience in constructing a large number of them. A new couple was used for each muscle, in order to avoid injury due to roughness and unevenness in the used ones. The wires were so thin, and the couples so smooth and clean, that they could be stuck through the tissue with a minimum of injury, especially if the muscle was stiffened by pulling while the wire was sewn in. Moreover, their lightness and flexibility prevented them from dragging on the muscle and straining it when it contracted. It was found quite unnecessary to insulate them. In order to ensure that the junction lay and remained right in the interior, the couple was provided with a little cross-piece of the same wire, which was soldered on the constantan wire about 3 mm. from the junction ; this cross-piece rested against the surface of the muscle with the junction inside.

The muscle was mounted in the frame shown in fig. 1. This was insulated with bakelite varnish and held in a rubber stopper by its brass tube. Four copper leads thickly insulated with bakelite were led up the side of the tube, two for the thermocouple, two for the stimulating electrodes. The frame, tube, copper leads and rubber stopper were all baked at 120° to harden the bakelite and to make it waterproof. A glass cover with an inlet tube at the bottom was fixed on the stopper.

The copper leads to the galvanometer ended in two thick pieces of copper heavily tinned. To these the thermocouple was soldered. They were heavy enough to prevent any rise of temperature of the cold junctions by conduction of heat along the wire. A bakelite clamp held the femur and a straight wire was tied to the Achilles tendon and led up the tube. Great care is necessary in fixing bone and tendon, or they may break, tear or slip under the relatively enormous tension (up to 2 kg.) developed in a tetanus.

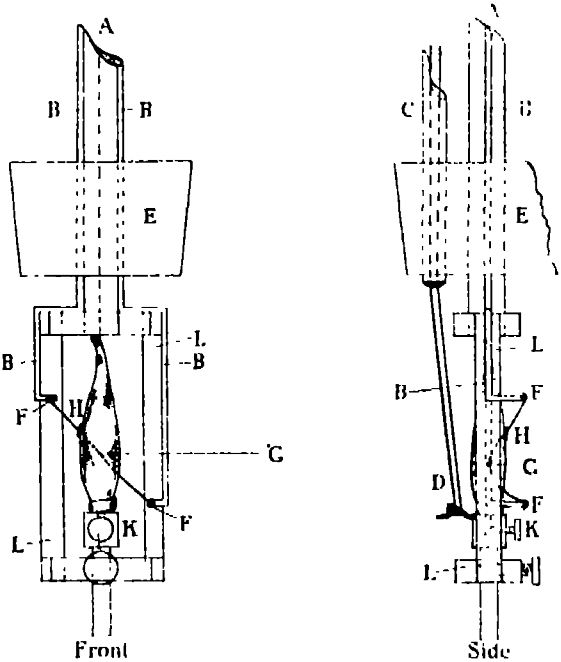

FIG. 1.—Frame for gastrocnemius-sciatic preparation with single thermocouple. A, brass tube. B, copper leads from thermocouple to galvanometer, fixed and insulated with bakelite varnish. C, tube carrying wires to silver nerve-electrodes D. E, rubber bung. F, "cold" junctions, where thermocouple is soldered to copper leads. G, "hot" junction inside muscle. H, cross-piece soldered to thermocouple, resting against muscle surface to keep junction G in position. K, clamp to hold femur. L, brass frame.

When a thermocouple was to be inserted it was tested by a magnet to find the iron end. This was threaded first as the cross-piece was soldered to the constantan. Its end was cut on the slant by a pair of scissors to give a sharp point, and this was thrust boldly into the belly of the muscle, held rigid under strong tension. The couple was drawn through the muscle until the cross-piece came in contact with the surface. The junction was then 3 mm. inside. The ends of the couple were soft soldered to the tinned copper terminals with a little soldering iron. The constantan end of the couple was then bent so as to exert a gentle forward thrust, tending to keep the cross-piece well home.

The sciatic nerve was drawn backwards and placed over silver electrodes on the side of the frame opposite to the thermocouple.

This part of the preparation took only a few minutes. It was now necessary to bring the whole arrangement to a constant temperature. The cover was placed in position on the stopper and connected by a rubber tube to a reservoir of oxygenated Ringer's solution, kept in the water-bath. The chamber was placed in the bath, the solution run into it, and oxygen bubbled through. Within 10 minutes the solution could be removed and the experiment begun.

The wire from the tendon was hooked to an isometric lever arranged to write on a drum. The lever was carried on a heavy stand, movable vertically by a screw, so that the initial tension could be adjusted.

The wires to which the thermocouple was soldered were connected by copper terminals to the galvanometer, and the deflection due to a small exactly known e.m.f. (1 to 5 microvolts) thrown into the thermocouple-galvanometer circuit was measured.

The muscle was usually in air, in the earlier experiments, however, in nitrogen. The diffusion of oxygen is so slow into the interior of a muscle so large (about 1 g.) that no appreciable recovery can be expected there, and it was thought better at first to avoid partial recovery occurring in the outer layer only, by removing oxygen altogether; later, however, similar results were obtained in air. For all the experiments recorded here large Hungarian frogs (*R. esc.*) of the same batch were employed. These reached England at the end of May and were used in June and July.

The character of the galvanometer deflection caused by stimulation is quite unlike that obtained with an ordinary thermopile. During the actual production of heat the galvanometer moves out usually rather rapidly; then it stands still for quite a long time, and then very slowly returns. The slowness of cooling is due to the fact that the cold junctions do not warm up and to the absence of contact with any good heat conductor; the thermocouple is much too small to have any considerable cooling effect; heat loss is almost entirely through the air, and from such large muscles is slow. So gradual is the return that one has to wait several minutes before another reading can be taken. The slowness, however, has the advantage of enabling the instrument to give correct readings, even for quite long intervals (up to 30 seconds) of stimulation. It is thus possible to obtain greater accuracy by working with groups of twitches, usually with 10; at high temperatures these generally occupied 6 to 9 seconds; at low temperatures 18 to 23. Moreover, the heat production for 25-second tetanus (at 5° C.) could be read directly on the scale without analysis or correction.

No attempt has been made to study either the recovery heat or the delayed anaerobic heat. The oxygen supply for the former would be inadequate in the large muscles used, while for both accuracy would be unattainable for the following reason. The gastrocnemius contains a fair amount of connective tissue, and if the thermojunction lay in some of this it would not be warmed immediately but only slowly by conduction. There is no error in the maximum reading, since in the 30 seconds or more before the galvanometer begins to return temperature equalisation within the muscle occurs. It is noticeable, however, that the character of the outward deflection differs considerably from one muscle to another; in some cases it is rapid, in other cases rather slow. Not only connective tissue but slight local injury can have this effect. In pushing the wire through the muscle undoubtedly a few fibres are injured, more on some occasions than on others. Again, no error in the maximum reading occurs, since there is plenty of time for the heat to conduct through the thin sheath of injured tissue; but the speed of deflection may be rather considerably affected. The existence of these variations due to conduction makes it quite impossible to separate delayed from initial heat, and the quantity recorded in the present experiments is the total heat in 15 to 30 seconds; the delayed anaerobic heat presumably supplies a small fraction of this, an unknown amount scarcely large enough to affect the result.

Since the heat capacity of the thermojunction is negligible, and since the rate of heat loss is so small, the maximum deflection is a measure of the rise of temperature of the muscle. The following example illustrates the method of calculation.

A gastrocnemius weighing 1·10 g., of density 1·0485, and therefore (see below) containing 17 per cent. solid and of specific heat 0·88, was mounted with a single thermocouple. 2·49 microvolts gave a deflection of 320 mm. The thermo-e.m.f. of the metals was 54·3 microvolts per 1° C. The *rise of temperature* per 1 mm. deflection therefore was:—

$$\frac{2\cdot 49}{320 \times 54\cdot 3}\ ^\circ\text{C.}$$

Hence the *heat* per 1 mm. deflection was :—

$$\frac{2\cdot49 \times 0\cdot88 \times 1\cdot10}{320 \times 54\cdot3} \text{ calorie,}$$

or

$$\frac{2\cdot49 \times 0\cdot88 \times 1\cdot10 \times 42650}{320 \times 54\cdot3} = 5\cdot92 \text{ g. cm.}$$

In this experiment (record in fig. 9) 10 twitches at 0·9-second intervals gave 280 mm. deflection and 6120 g. total tension. The muscle was 3·7 cm. long. The total heat therefore was $280 \times 5\cdot92 = 1660$ g.cm., and $Tl = 6120 \times 3\cdot7 = 22600$. Hence,

$$Tl/H = 13\cdot6.$$

In tetani the sensitivity was reduced by putting a resistance in the circuit, otherwise the procedure was the same. For further records see figs. 6 and 7.

For this calculation it is necessary to know the specific heat of the muscle. In the absence of reliable data in the literature the measurements given in the next section were made.

The Specific Heat of Muscle.—As Bürker remarks (1910) it is curious that a quantity so important as the specific heat of muscle should be so imperfectly known. Adamkiewicz (1875) found a value of 0·77 and Rosenthal (1878), employing the Bunsen ice-calorimeter, one of 0·825. Rosenthal recognised that the determining factor in the specific heat of muscle is its water content, and he estimated that the solid material of muscle, when perfectly dry, would have a specific heat of 0·30; his value of 0·825 would correspond to 75 per cent. water, 25 per cent. solid. The fact that practically all the water of muscle is "free" in the physico-chemical sense (Hill, 1930, *a*) encourages one to believe that Rosenthal was right in his assumption that the heat capacity of muscle is the sum of the heat capacities of its water and its solids, at any rate with sufficient accuracy for the present purpose. It was obviously desirable, however, to redetermine the actual value.

A spherical Dewar flask of about 220 c.c. capacity was employed, together with a standard thermometer, an insulated heater resistance of constantan wire, a battery of accumulators and a potentiometer to measure their e.m.f. A current was passed through the resistance for a known time, sufficient to cause a rise of temperature of about 10° C. The water equivalent of the flask was determined by the same method and found to be 8·7 g. The specific heat at 20° C. of frog's Ringer's fluid (containing 0·71 g. solid in 100 g. H_2O) was found to be 0·9933. The following results were obtained with muscle from Hungarian frogs (*R. esc.*) containing 17·3 per cent. of solid (determined by prolonged drying at 120° C.) :—

(A) 59·87 Ringer + 59·57 g. muscle. E.m.f. = 20·247 volts. Time = 600·0 seconds. Corrected rise of temperature 22·115 − 13·945 = 8·17°. Heat 120·44 20°-calories. Specific heat of muscle 0·877.

(B) Same continued, corrected rise of temperature 30·435 — 22·24 = 8·195°. Specific heat of muscle 0·865. (This value has not the same weight as (A), since the resting heat production at the mean temperature of 26° was much greater than at the mean temperature of 18° C., and allowance for this was not very accurate.)

(C) Same muscles next day when dead, corrected rise of temperature 27·32 — 19·16 = 8·16°. Specific heat of muscle 0·893.

Allotting weighting factors 3. 1 and 2 to (A), (B) and (C) respectively, we obtain a mean value of 0·880.

The experience, however, of these three determinations made it clear that (*a*) the resting heat production, which varies with temperature, and (*b*) the fact that the muscle had to be used as lumps suspended in Ringer's solution, and not as a fluid capable of being properly mixed by stirring, might prevent the highest degree of accuracy from being attained. It was decided therefore to determine the specific heat of ox blood. From the physico-chemical standpoint the constituents of blood are almost identical with those of muscle, and it is very unlikely that the specific heats of the solids in the two cases are considerably different. The same method of electrical heating was used, but the absence of resting heat production and the possibility of complete mixing made the results more consistent and accurate. The blood was partially centrifuged to increase its total solid about to that of muscle, and in both samples the total solid was determined by drying at 120° C. The following results were obtained :—

Blood A—19·5 per cent. solid, specific heat 0·8655.

Blood B—22·32 per cent. solid, specific heat 0·8497.

Blood B—22·32 per cent. solid, another determination of the specific heat with another flask, 0·8472.

From these three values the specific heat of the solid constituents can be calculated : 0·316, 0·329, 0·319 respectively. Taking 0·32 as the mean, and recalculating the specific heat of the blood from its solid content, we find in the two samples :—

(A) $0 \cdot 8043 + 0 \cdot 1957 \times 0 \cdot 32 = 0 \cdot 867$ (observed 0·8655).

(B) $0 \cdot 7768 + 22 \cdot 32 \times 0 \cdot 32 = 0 \cdot 848$ (observed 0·8497 and 0·8472).

Applying this to the case of muscle containing 17·3 per cent. of solid the specific heat should be $0 \cdot 827 + 0 \cdot 32 \times 0 \cdot 173 = 0 \cdot 882$. The mean value observed directly for muscle was 0·880 ; within the limits of error these may be regarded as identical. We may conclude, therefore, that the specific heat of muscle or blood can be calculated from its total solid content, assuming water to have a specific heat of unity (at 20° C.) and the solid a specific heat of 0·32. The following table may be useful :—

Solid (per cent.)	25	24	23	22	21	20	19	18	17	16	15
Specific heat (20° C)	0·830	0·837	0·844	0·850	0·857	0·864	0·871	0·878	0·884	0·891	0·898

The average solid content of the frog's muscle used in the present investigation was about 17½ per cent., so that its specific heat was about 0·88. This value has been assumed whenever the water content was not directly measured.

A Rapid Method of Determining the Total Solid in Frog's Muscle.—Since the specific heat of muscle depends so largely on the amount of water it contains, and since the method of measuring this by weighing, drying and weighing is time-consuming, the relation between the density of and the total solid present in muscle was determined. The measurement of the density is simple and rapid, when carried out as follows. The muscle was hung by a very fine enamelled copper wire to the hook of an air-damped Bunge balance and weighed. A beaker of Ringer's fluid (0·71 g. total salt in 100 g. H_2O) was placed on a bridge above the balance pan, and the muscle, still hanging from the hook but immersed in the solution, was weighed again. Finally the wire was cut as near the muscle as possible, below the Ringer's fluid, and a third weighing made. The three weights enable the density of the muscle to be calculated as a multiple of that of the solution. The effect of surface tension, as well as the weight of the wire, is eliminated by the third weighing. The following example illustrates the calculation.

Gastrocnemius of Hungarian *R. esc.*

	g.
(1) Weight, hanging by wire in air	1·300
(2) Weight, hanging by wire in Ringer's fluid	0·0652
(3) Weight of wire cut off below surface of solution	0·0043
(4) Difference between (2) and (3) = weight of muscle in solution	0·0609
(5) Difference between (1) and (2) = weight of same volume of Ringer's fluid	1·235

$$\frac{\text{Density of muscle}}{\text{Density of Ringer's fluid}} = 1 + \frac{0\cdot0609}{1\cdot235} = 1\cdot0493.$$

Solid, determined by drying at 120° C., 17·1 per cent. Hence each 1 per cent. solid adds 0·00288 to the relative density.

Two such comparisons, each the mean from two muscles of the same frog, were made by the method described. As a check three comparisons were made with a pyknometer, a given weight of muscle being placed in the bottle which was then filled with Ringer's fluid and weighed. The following five results were obtained for the effect of 1 per cent. solid on the relative density, the percentage of solid being determined by drying at 120° C. :—

(A) Weighing in Ringer's fluid : 0·00287, 0·00288.
(B) Pyknometer : 0·00286, 0·00287, 0·00283.

The mean of all is 0·00286, from which, knowing the relative density by weighing, we can immediately calculate the percentage of solid and the specific heat, for example :—

$$\text{(Density of muscle)} = \text{(density of Ringer's fluid)} \times 1{\cdot}0540.$$

$$\text{Percentage solid} = \frac{0{\cdot}0540}{0{\cdot}00286} = 18{\cdot}8.$$

$$\text{Specific heat} = 0{\cdot}812 + 0{\cdot}188 \times 0{\cdot}32 = 0{\cdot}872.$$

The specific heat was determined in this way in a few experiments, but since, in the batch of Hungarian frogs used, it always came to be about 0·88 this value was assumed for the remainder.

This method of determining the total solid might be usefully employed in experiments on the osmotic swelling, or shrinkage, of muscle. It seems to be rather accurate.

The Value of Tl/H *in Sartorii.*—The chief purpose of the present investigation was the determination of Tl/H in the twitches of gastrocnemii. It was desirable, therefore, to have for comparison a few observations on sartorii, made on the same batch of Hungarian frogs with the usual method. The values of Tl/H were measured (*a*) in twitches, and (*b*) in 2-second and 5-second-tetani, all at room temperature. Three different thermopiles were used. The calibrating electrodes were either fixed or tied round the muscle at suitable points. The length l is the distance between the calibrating electrodes, since it is in this position alone of the muscle that the heat is measured ; l was 20 to 30 mm. ; between the electrodes the muscles were of very uniform section.

Particular care was taken to make the contractions as isometric as possible since (at any rate in twitches) permitting a muscle to do work in extending its connections to a lever may cause a considerable diminution of Tl/H. The muscle was connected by a wire to as good an isometric lever as was available. It was soaked in oxygenated phosphate-Ringer's fluid (p_H 7·2) for some time before the observations began ; these were made in oxygen, and intervals of 2 minutes between twitches, and of about 15 minutes between tetani, were given to allow recovery to go on. With all these precautions rather high values of T*l*/H in twitches were obtained. At the end of an experiment the muscle was killed by chloroform and calibrated by the new method described below.

As the initial length of a muscle is altered the values of T and H in a twitch change, and pass through maxima at lengths corresponding to a quite small initial load (see Hill, 1925). These lengths are nearly, if not quite, the same, and T*l*/H also passes through a maximum at about the same length. In all the experiments, therefore, trials were carefully made to find the length at which T and H were greatest, and the rest of the observations were made at this length.

The results of six good experiments (July) are given in Table I. The tensions, even in the 5-second tetani, were well maintained, owing to the use, as stimuli, of alternating condenser discharges at a frequency only just sufficient for summation.

Table I.—T*l*/H in Twitches and Tetani of Sartorii of Hungarian *R. esc.*

Number	1	2	3	4	5	6
Temperature 0° C.	20·7	16·7	16·6	18·25	18·5	18·85
T*l*/H, twitches	7·1	6·6	5·4	8·6	5·9	7·2
T*l*/H, 2-second tetanus	0·465	0·525	0·473	0·80	0·566	0·631
T*l*/H, 5-second tetanus	—	0·249	0·445	0·366	0·274	0·208
T*l*/H, 2-second tetanus	—	0·565	0·226	—	0·618	0·653

The mean value of T*l*/H in twitches is 6·8, which is not far from the mean value (6·16) previously obtained (Hill, 1928, *b*) for the sartorius of *R. temp.* or Dutch *R. esc.* That it is slightly higher is probably due to the precautions taken (*a*) to work at the optimal length, and (*b*) to ensure an inextensible connection to the lever.

The observed values of T*l*/H in the tetani are given by large circles in fig. 8 below. It was shown by Feng (1931) that the effect of temperature on H/T*l* in a tetanus may be regarded as due simply to a change in the time scale of the contraction; x second tetanus at 20° C. gives the same value of H/T*l* as $x \times 3 \cdot 1$ seconds at 10° C. or as $x \times (3 \cdot 1)^2$ seconds at 0° C. The times, therefore, viz., 2 and 5 seconds, have been "reduced" to 0° C. by multiplying by $(3 \cdot 1)^{\theta/10}$ where θ° C. was the temperature of the experiment. The values of H/T*l* are plotted against the "reduced" time. They are somewhat widely scattered—Hungarian *R. esc.* give more variable results than English *R. temp.*—but they lie around Feng's mean curve, which is shown for comparison. So far as a difference can be detected from so few experiments the larger animal seems (as we should expect) to be the slower, its values for H/T*l* tending to lie below Feng's curve for *R. temp.*; a 10 to 15 per cent. reduction in the

" time scale " on which the large circles have been plotted would centre them accurately on Feng's curve for the smaller animal. Not enough experiments, however, have been made to be sure of this point, but it is certain that the curve for sartorii lies well above that for gastrocnemii to which we will refer later.

Calibration by Condenser Discharges.—The method of " calibrating " a sartorius or similar muscle lying on a thermopile by passing a warming current through it (see Hill, 1913, p. 455 ; 1928, *a*, p. 131) can be greatly improved and simplified by adopting Bozler's suggestion (1931) of using condenser discharges. His arrangement, too, can be somewhat improved in detail, as follows.

The principle of Bozler's calibration is simple. The energy of a condenser of capacity C charged to potential E is, in electro-magnetic units, $\frac{1}{2}CV^2$, or, expressing C in microfarads and E in volts, the energy is $5CV^2$ ergs. If the condenser be discharged along wires of negligible resistance through a muscle of resistance R, by electrodes placed upon it, the whole of this energy is liberated in the muscle as heat. The duration of the discharge can readily be calculated ; the fraction of the original charge remaining after t seconds is $e^{-t/RC}$, the fraction of the original energy $e^{-2t/RC}$. Thus if $C = 0 \cdot 5$ mfd. $=$ 5×10^{-7} farad, which has been found to be a suitable capacity, and $R = 10{,}000$ ohms, which is a usual resistance for muscles used in myothermic experiments, the charge is reduced to 1 per cent. in a time given by $e^{-200t} = 0 \cdot 01$, from which $t = 0 \cdot 023$ second ; the energy is reduced to 1 per cent. in half this time, viz., in 0·0115 second. The liberation of energy, therefore, may be regarded as " instantaneous."

By employing a relatively high potential and a low capacity the effects of polarization at the electrodes may be made very small. The single twitch of a muscle gives about 0·003 calorie per gram, *i.e.*, in a muscle of 200 mg. about 0·0006 cal., 25·6 g. cm., 25,100 ergs. A condenser of 0·5 mfd. charged to 100 volts yields 25,000 ergs. on discharge. A 2-second tetanus at 20° C. gives 0·06 cal. per g., *i.e.*, in the same muscle about 500,000 ergs. A 0·5 mfd. condenser charged to 450 volts will yield this in one discharge ; 450 volts, however, is inconveniently high, and it is better, if an " instantaneous " heating is required, to use a condenser of higher capacity. Usually, however, a calibrating heat production of about the same duration as the tetanus is best for accuracy. Three charges and three discharges of 180 volts of a 0·5 mfd. condenser give $30 \times 0 \cdot 5 \times 180^2 = 486{,}000$ ergs., and these can easily be distributed by hand over a 2-second interval ; the charge and discharge being in opposite directions tend to neutralise polarisation. If required, moreover, a commutator can be used, giving discharges only, or charge and discharge alternately, during a time determined by a revolving contact breaker. The arrangement shown in fig. 2 is simple and can be employed for stimulation as well as calibration. Its use is explained in the legend.

The condensers employed for calibration in the present work were of about 0·5 and 1·0 mfd. Their capacities were accurately determined to within 0·001 mfd. by comparison with a known standard condenser. The condensers should be good ones, preferably of mica, so that they hold no residual charge. The potentials were measured with a high resistance Weston voltmeter.

As stimuli for single twitches, condenser discharges, whether to nerve or muscle, are much more easily controlled than induction shocks. For tetani the arrangement shown in fig. 2 with the commutator is far superior to an induction coil. The individual shocks can be accurately graduated, and delivered at such a speed that summation is only just complete. It is very important, if a tetanus is to be well maintained, that the frequency should not

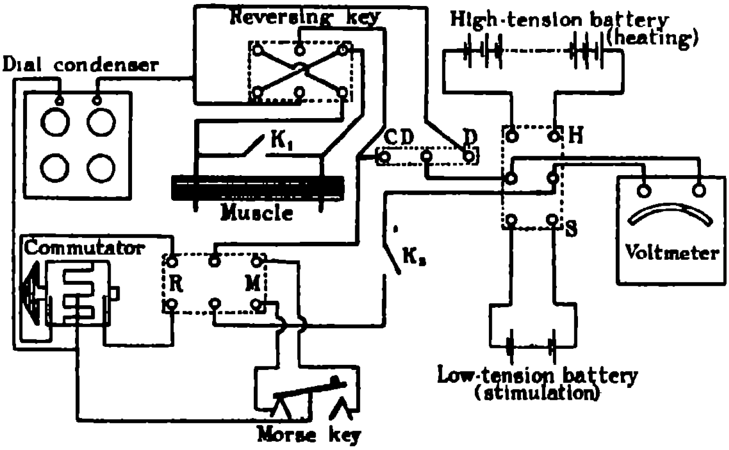

FIG. 2.—Electrical connections for stimulation, or calibration, by condenser discharges. H–S and R–M double-pole throw-over switches. CD–D single-pole throw-over switch. When contact is made to H, the high tension battery is connected for heating; when to S, the low tension battery for stimulation. The reversing key is employed to give the best direction for stimulation. When contact is made to D, condenser discharges alone pass through the muscle; when to CD charges and discharges pass through it. When contact is made to M, the Morse key is in circuit, for discharge, or charge and discharge, by hand; when to R the commutator is in circuit for regular series of discharges (or for alternating charge and discharge). The commutator, if desired, can be replaced by a simple rocker, like a Morse key, driven mechanically. The dial condenser used was adjustable from 0·001 mfd. to 4·11 mfd.; this is convenient, but a few good condensers, *e.g.*, 0·5, 0·1 and 0·05 mfd. are sufficient for nearly all purposes. K_1 and K_2 are keys on a Lucas revolving contact breaker, opened at any desired interval to allow the stimulating, or calibrating, current to pass during that interval. K_2 is closed when the Morse key is used.

be too high; at 5° C. with frog's muscle it need not be more than 6 per second, at 0° C. 3 or 4 per second are sufficient. The speed of the commutator (or rocker) can be set to any desired value, and so with an adjustment of voltage and capacity a complete control of stimulation is obtained.

As Bozler points out (1931) this method is greatly superior to the old one, when employed for calibration in nerve-heat measurements. In the old one the very weak alternating current used for calibration had to be measured, which required extremely sensitive instruments. With the present method the energy in the condenser discharges is just as accurately known, however weak these may be; the capacity and the potential can be reduced as required, without diminishing the accuracy with which the energy is measured.

The Value of Tl/H *in Twitches of a Frog's Gastrocnemius.*—In an isometric twitch, as the length at which the tension develops is increased Tl/H increases at first, then attains a maximum, and then decreases again. T and H have maxima at about the same length, and the maximum of Tl/H is rather blunt (see Hill, 1925). The greatest value, therefore, of Tl/H is easily obtained by working with an initial tension which gives the greatest value of T; in the gastrocnemius, which is very inextensible, this is most readily found by raising the tension lever away from the muscle until the initial tension just begins to increase rather rapidly. All observations have been made at this length.

The Achilles tendon was very firmly tied to a strong straight wire leading up to the lever, which was as nearly isometric as could be made. It is very important in a twitch, if the maximum value of Tl/H is required, to make the contraction as isometric as possible. The lever was calibrated over its entire range of movement, and millimetres on the drum were read off as grams weight on a curve. Condenser discharges were employed as stimuli to the sciatic nerve, usually 4 volts, 0·03 to 0·05 mfd.; these were well in excess of maximal but not so great as to produce repetitive response. Usually 10 twitches were given at intervals of 0·7 sec. (or at low temperatures, in the neighbourhood of 5° C., at intervals of 2 to 3 seconds), the total initial heat being measured by the galvanometer deflection, and the tension recorded on a moving drum. In some of the early experiments single twitches were given, but greater accuracy is attained by giving at least 5; it is possible indeed to record the heat in as many as 30 twitches, in a single galvanometer deflection.

The experiments were in three series, all on the same batch of Hungarian frogs. The first series, on 20 muscles, I made myself between June 8 and July 9 all at room temperature (about 20° C.). The values of Tl/H were as follows, T being in grams weight of tension developed, l being muscle length in centimetres and H initial heat in gram centimetres :—

10·7, 14·1, 11·7, 11·8, 12·3, 12·3, 11·8, 10·7, 12·0, 16·0, 14·6, 13·6, 14·3, 15·3, 14·2, 14·4, 17·6, 15·9, 16·7, 15·7 ; mean 13·8.

The next series was made independently at room temperature by Dr. McKeen Cattell on the same frogs. His values (July 7 to July 10) were :—

16·7, 11·6, 11·8, 10·8, 12·7, 13·1, 12·6, 11·3, 12·6, 13·5, 11·8, 11·0, 15·3, 14·7, 13·3 ; mean 12·9.

A third series was made by Dr. Cattell at about 5° C., the values were :—

14·4, 12·0, 12·5, 15·1, 12·9, 15·1 ; mean 13·7.

As usual we find that Tl/H in a twitch is independent of temperature, and it is satisfactory that two different observers should obtain results so close to one another. The mean of all values is 13·4 and the root mean square deviation of a single reading is 1·8 ; thus, assuming no consistent error to have occurred, the probable error of the mean of the 41 readings is only 0·2, so that we may regard the value 13·4 as reasonably certain.

This value of Tl/H is just twice as high as that obtained in the present research with sartorii of the same batch of frogs. It is most unlikely that the difference is due to any inherent difference between the fibres of the two muscles. The measurement of the length of a gastrocnemius is certainly rather arbitrary. It is simple enough to stretch it to a "standard" length, *i.e.*, until the tension just begins to rise rapidly, after which it becomes very inextensible. The difficulty lies in deciding on the points on it from which to measure. The upper end, where muscle joins bone, is fairly definite ; the difficulty occurs at the lower end ; where does muscle cease and tendon begin ? If, however, they examine it closely with a lens two observers will usually agree within 1 mm. as to the point where muscle finally merges into tendon, and if this point be marked with ink the measurement is easy. It is important, in view of the comparison (see Discussion below) of the present results with his chemical ones (1931), that Dr. R. Lundsgaard and I found that we agreed within 1 mm. in estimating the length of a gastrocnemius ; Dr. Cattell and I similarly agreed ; 1 mm. is only 3 per cent. of the length, which (as a random error) is negligible in comparison with the mean deviation of a single determination of Tl/H. Thus, if it be agreed to measure from the point where the round end of the muscle joins the bone to the point where muscle finally merges into tendon, no important random error is made. It remains to consider whether any consistent error arises. In the case of the sartorius the measurement of l is practically free from error, since the portion of muscle considered is the nearly uniform piece between the electrodes, which are at a definite distance apart.

The real explanation of the difference between Tl/H for gastrocnemius and sartorius is almost certainly that in the former the fibres (or groups, bundles or chains of fibres) do not run, on the average, more than half the length of the muscle. The rest of the length is made up by tendon. Indeed, the shape of the gastrocnemius implies that many of the fibres run diagonally, thus ensuring a greater development of tension for a given weight and length, at the expense of a smaller possible shortening. In 1913 I estimated that the area of the tension-length curve of an excited sartorius is about 1/6·9 of the product Tl, where T is the tension developed at the " resting " length l. In 1921 Meyerhof in one experiment each on an adductor, a semimembranosus, a gracilis and two sartorii found an average factor of 1/8·5. For gastrocnemii he found an average factor of 1/13·4. In 1923 Fenn discussed the difference and concluded (with me) that it is due to the diagonal arrangement of the fibres of the gastrocnemius. In 1924 Mashimo confirmed this conclusion by taking a strip of the gastrocnemius containing parallel fibres; for this he found the area of the tension-length curve, in a tetanus of 0·6 second at room temperature, to be on the average 1/5 Tl, where l was the length at which maximal tension T was developed. In similar experiments on a strip of sartorius he found an average factor of 1/4. The absence of any considerable difference between the two muscles, when parallel fibres only were compared, showed that the diagonal arrangement of the fibres in the whole gastrocnemius was the real cause of the difference normally observed.

The considerable differences, however, found by various observers for the ratio Tl/A, where A is the area of the tension-length curve, made it desirable to reinvestigate the matter with greater precautions. This has been done, as described in the next section, with the result that Tl/A has been found in gastrocnemii to be on the average almost exactly double its value in sartorii, thus confirming the conclusion of the myothermic measurements that l in the gastrocnemius should be taken as half the measured length of the muscle.

The Ratio Tl/A.—For clearness of definition, let l be the length of a muscle at which it develops the maximum tension in a single twitch; l is not far from the resting length. Let T be that maximum tension. Let A be the area of the tension-length curve (for single twitches), measured up to but not beyond that length l. It is required to compare Tl/A for gastrocnemii and sartorii of the same, or similar, frogs.

The experiments were made at room temperature, the sartorii (used in pairs) being stimulated directly, the gastrocnemii (used singly) through their nerves. Condenser discharges were used as stimuli, about 4 volt, 0·1 mfd. for sartorii, 4 volt, 0·03 mfd. for gastrocnemii. The muscles were very carefully prepared, rigidly held, and connected by straight wires to a good isometric lever writing on a drum. The lever was held on a heavy Palmer stand, and so arranged that it could be screwed up or down 1 (or 1½) mm. at a time with accuracy. The muscle was first stretched well beyond the length for maximum tension development, and given a few preliminary stimuli. Shocks were then applied in pairs at each length, the interval between the two being 6 seconds; the lever was then screwed down, releasing the muscle by 1 (or 1½) mm., and after 12 (or 18) seconds two more shocks were given at the new length, once more with an interval of 6 seconds. Again the length was diminished by 1 (or 1½) mm. and after another 12 (or 18) seconds two more shocks were applied.

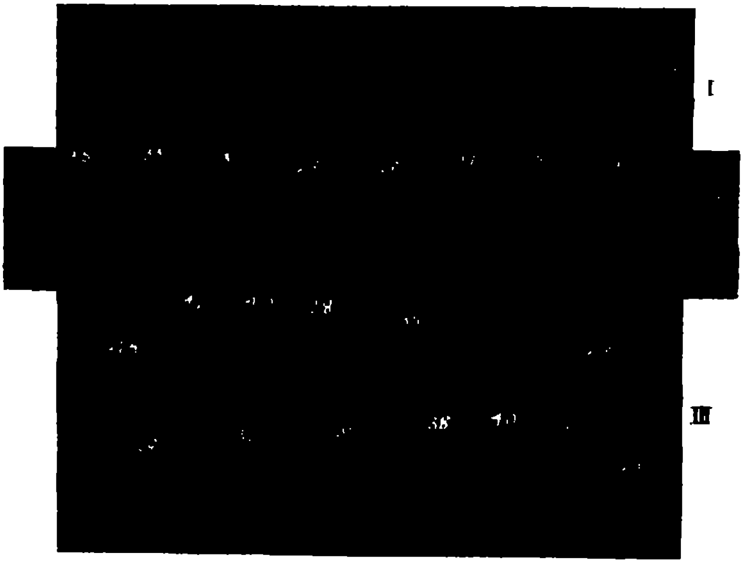

FIG. 3.—Records from gastrocnemii (I and II) and sartorius (III). Maximal twitches. The numbers represent length in millimetres. Four twitches at each length, two in descending and two in ascending series. The second twitch only of each pair is measured, and the mean of one in the descending and one in the ascending series is plotted in fig. 4 against length. 6 seconds between twitches of a pair; 18 seconds between pairs, during which the length was changed. Note that the sartorius record (III) was made in two halves, the descending and the ascending series respectively.

The procedure was continued until practically no tension was developed, and then reversed, pairs of twitches being recorded at the same intervals with increasing length. When the original length was reached the experiment was at an end. The tensions were registered on a slowly moving drum. Records on the two gastrocnemii of one animal are given in fig. 3, and for comparison, below, the record from the sartorii of the same animal. Note that the gastrocnemius records are each in a single portion; for the sartorius, tensions with decreasing lengths are above, with increasing lengths below. It is evident at once how much more shortening the sartorius can undergo, and still develop tension, than the gastrocnemius.

In calculating the results, the second twitch only of each pair at any length was measured, but since each length was reached twice, once in the descending and once in the ascending series, there were two values of the tension developed at every length, and the average of these was taken. By this means any progressive change in the muscle due to stimulation, whether "treppe" or

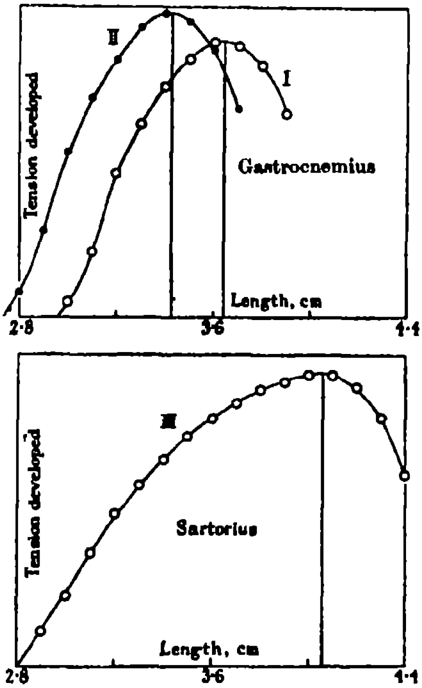

FIG. 4.—Mean tensions measured from records of fig. 3 and plotted against length. I and II, gastrocnemii; III, sartorius pair; all of the same animal (Hungarian *R. esc.*). Vertical lines are drawn for each curve to show the maximum tension T. If *l* be the length for maximum tension, and A the area of the curve to the left of the maximum tension, T*l*/A has the following values: (I) 8·75, (II) 8·2, (III) 4·99. Note that the curves for gastrocnemius and sartorius are almost precisely similar in shape, but much wider in the case of sartorius, corresponding to the greater fibre-length.

fatigue, tends to be eliminated. In fig. 4 the tensions measured off from the records of fig. 3, and averaged as described, are plotted as a function of the length. The value of *l* is taken as the length at which the tension is a maximum. T is that tension, and the area of the tension-length curve is measured to the left from the maximum tension. In the top record (fig. 3) (curve I, fig. 4) the ratio T*l*/A is 8·75 ; in the middle record (fig. 3) (Curve II, fig. 4) it is 8·2 ; mean (say) 8·5 for the gastrocnemii. In the bottom record (fig. 3) (curve III, fig. 4) T*l*/A is 4·99 for the two sartorii. The one ratio is 1·7 times the other in this particular animal. This is less than the mean, which is 2 (see below) ; probably individual gastrocnemii differ from one another in the average slant of their fibres, certainly they differ in shape, some being relatively much thicker than others. This may be one cause of the observed variation of T*l*/H.

Many such experiments have been made, of which the results are given in Table II. The mean for the gastrocnemius is 9·8, and for the sartorius 4·9. The ratio is 2·0. The ratio of T*l*/H for gastrocnemius to T*l*/H for sartorius is 13·4/6·8 = 1·97, almost exactly the same. Thus A/H is the same for gastrocnemius as for sartorius, where A is the area of the tension-length curve, and the reason why T*l*/H is twice as great for the former as for the latter is

Table II.—The Ratio T*l*/A in Twitches of Gastrocnemius and Sartorius.

Number of frog :	1.	2.	3.	4.	5.	6.	7.
T*l*/A, gastrocnemius	8·2, 8·3	8·2, 8·75	8·4, 8·4	8·9	10·0	—	9·0
T*l*/A, sartorius	4·83	4·99	4·66	—	—	4·87	—

Number of frog :	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.
T*l*/A, gastrocnemius	8·4	9·2	9·75	10·1	9·9	—	—	—	—	—
T*l*/A, sartorius	—	—	—	—	—	4·8	4·6	6·7	4·72	4·45

Number of frog :	18.	19.	20.	21.	22.	23.	Mean.
T*l*/A, gastrocnemius ..	—	10·7	10·0, 10·7	12·8	11·8	10·5, 13·6	9·8
T*l*/A, sartorius	4·96	—	4·05	—	—	—	4·9

Note.—In five experiments on short tetani of a sartorius at 0° C. Hartree and Hill (1928, p. 12) found values of T*l*/A, 4·9, 4·7, 6·0, 5·6, 4·9, mean 5·2. From six experiments (sartorii, 0·1-second tetanus) at 9° and 15° C., Hill (1925, p. 257) constructed a mean curve, relating tension developed to length, which is very similar to that of fig. 4 and allows a value of T*l*/A = 4·65 to be calculated. Both these values are in close agreement with the one given in this table for sartorii, indeed their mean coincides with it.

that l is twice the average length of the fibres, or of the bundles of fibres in parallel.

The factor used by Meyerhof to reduce the length of a gastrocnemius to the average length of its constituent fibres is 0·69 (see Meyerhof, 1930, p. 233). The value found here by two independent methods, viz., 0·5, differs so considerably from Meyerhof's that it will be necessary to recalculate his isometric coefficients whenever they have been measured in gastrocnemii and compared with other muscles. It is most unlikely that his frogs differ much in this respect from the batch used in the present research. It may be that there is a small consistent difference between our measurements of the length of a gastrocnemius, but it cannot be as large as the difference between our factors 0·5 and 0·69; and Dr. Lundsgaard, who has worked with both of us, agreed with my estimates of the length. Thus in comparing the isometric coefficients for lactic acid and phosphagen observed on gastrocnemii with the isometric coefficient for heat the value 13·4 found here should be assumed for the latter, and in comparing gastrocnemii and sartorii the lengths of the former should be multiplied by 0·5 before the comparison.

The Effect of Activity on the Ratio Tl/H in a Twitch.—In 16 experiments Tl/H was measured in successive series, sometimes with 2-second tetani interposed. Taking a 2-second tetanus as equivalent to 20 twitches, dividing the value of Tl/H by that observed in the first series of all, and plotting the relative value of Tl/H so calculated as a function of the number of preceding twitches, we obtain fig. 5. The points are rather scattered, but that is partly due to the large scale of the figure. It is clear, however, that there is a definite increase in Tl/H with the amount of preceding activity. There is no fatigue, properly so called, after 60 twitches; each twitch gives an initial heat of 0·003 to 0·004 cal. per gram., 60 twitches therefore about 0·2 cal. per gram, which is not more than one-third or one-quarter of the total heat the muscle is capable of giving out anaerobically before exhaustion. The twitches occurred in small groups, usually of 10, with several minutes' interval between, so that although no oxidative recovery was possible in the interior of the muscle, anaerobic recovery (*e.g.*, the partial restoration of phosphagen) can have gone on to the greatest degree possible. This increase of Tl/H with activity may be associated (1) with the change over from phosphagen breakdown to lactic acid formation as the source of energy for contraction, or (2) with the change of p_H.

The effect of previous stimulation on Tl/H in a twitch was investigated before (Hill, 1928, *b*, p. 165) on sartorii (English *R. temp.* or Dutch *R. esc.*) in

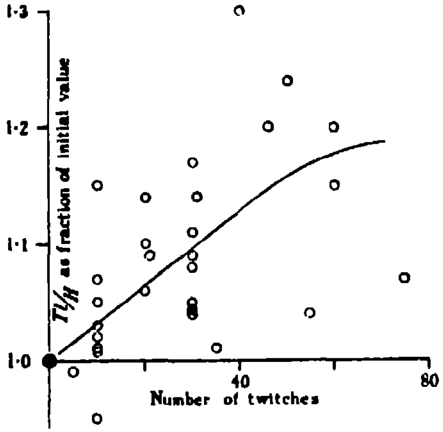

FIG. 5.—The increase of Tl/H in a twitch with preceding activity. Abscissa—the number of preceding twitches (or their equivalent, 2-second tetanus at 20° C. being assumed equivalent to 20 twitches). Ordinate: the value of Tl/H in a short series of twitches, expressed as a fraction (or multiple) of that in the first series of all.

Table III.—The Effect of Previous Activity on the Ratio Tl/H in a Twitch.

Number of experiment :	1.	2.	3.	4.	5.	6.	7.	8.
Number of twitches	5	10	10	10	10	10	10	10
Tl/H	11·8	12·3	11·8	12·0	16·0	14·6	13·6	11·8
Number of twitches	10	11	10	10*	10*	10*	20*	10
Tl/H	11·7	12·7	12·0	13·3	16·7	15·2	13·7	12·4
Number of twitches	....	9	11	—	10*	10*	20	10
Tl/H	—	13·4	12·5	—	18·4	17·5	14·2	13·5
Number of twitches	—	16	10	—	—	—	20	—
Tl/H	—	13·3	13·4	—	—	—	14·6	—
Number of twitches	—	19	20	—	—	—	—	—
Tl/H	—	15·2	14·2	—	—	—	—	—

Number of experiment :	9.	10.	11.	12.	13.	14.	15.	16.
Number of twitches	10	10	10	10	10	10	10	10
Tl/H	13·1	11·3	13·5	11·8	15·3	14·7	11·6	12·8
Number of twitches	10*	10	10	10	10*	10*	10	10
Tl/H	15·3	11·4	15·6	11·9	16·1	16·0	12·4	12·2
Number of twitches	10	10	—	—	10*	—	—	—
Tl/H	17·4	12·4	—	—	18·2	—	—	—

* Preceded by a 2-second tetanus.

Note.—All the experiments were at about 20° C. except the last two which were at 5° C. After each series, or tetanus, several minutes were allowed for the temperature to settle down.

nitrogen after previous soaking in phosphate Ringer's fluid at p_H 7·4 to 7·6. Very little change in Tl/H was found, even after considerable activity (indeed, up to three-quarters of total exhaustion). The earlier part of the range, however, was not specially studied, and it is difficult to detect in the older results any sign of the increase of Tl/H during the first 60 twitches found in the present experiments. Very likely it did not occur. The change of p_H during the earlier stages of activity is almost certainly different (*a*) in a sartorius previously soaked in well buffered Ringer's solution at p_H 7·4 to 7·6, and (*b*) in a gastrocnemius not soaked for long and because of its size almost unaffected by soaking; possibly the behaviour of Tl/H depends upon the initial p_H and the buffering of the muscle. It may be that the muscles of the large Hungarian frogs differ in some respect affecting the behaviour of Tl/H from those of the small English and Dutch ones. The increase of Tl/H shown in fig. 5 appears so regularly, though in somewhat variable amount, that it is difficult not to believe in its real existence; it may somehow be associated with the greater "spreading-out" of the twitches of the later series, which is much more obvious in the Hungarian frogs than in the English ones. The matter needs further investigation.

The Value of Tl/H *in Tetanic Contractions.*—Many experiments were made on gastrocnemii with tetanic stimuli. At the higher temperature (about 20° C.) these were all (except one) of 2 or 5 seconds' duration; at the lower temperature (about 5° C.) they were all of 10 or 25 seconds. Maximal condenser discharges were used as stimuli, at a frequency very nearly sufficient to cause complete summation (see figs. 6 and 7). By this means the tensions were better maintained. No experiment is included in which the tension fell off appreciably during the stimulus. In several experiments the tension developed was poor; these also were discarded.

The muscles were in all cases dissected some time before the experiment and kept in well oxygenated Ringer's fluid in a beaker and then in the thermocouple chamber. After thermal equalisation the Ringer's fluid was removed and the muscles given a few shocks for test purposes, and then in the earlier experiments one or more series of 10 shocks to allow Tl/H in twitches to be measured. A 2-second tetanus was then applied, followed either by more twitches or by further tetani. The results in Table IV were obtained (June 10 to July 1) on 2-second tetani at the temperatures named. The "reduced" time is calculated, according to Feng (1931), by multiplying the actual time by $(3\cdot1)^{\theta/10}$ where θ was the temperature of the experiment in question, and 3·1 is the temperature coefficient.

Table IV.

Temperature, ° C.	18	18·9	18	20·1	21·9
" Reduced time," seconds at 0° C.	15·3	17·0	15·3	19·4	17·9‡
Number of preceding twitches	10	14	16	19	13
H/T*l*, 1st 2-second tetanus	0·64	0·65	0·775	0·58	0·735
H/T*l*, 2nd 2-second tetanus	0·52	0·52*	0·71*	0·48†	0·695
H/T*l*, 3rd 2-second tetanus	0·40	0·40	0·58	—	0·64
H/T*l*, 4th 2-second tetanus	0·30	—	—	—	0·61
H/T*l*, extrapolated to rest	0·70	0·71	0·81	0·63	0·78
H/T*l*, twitches	0·081	0·083	0·068	0·059	0·070

* Preceded by 10 twitches.
† Preceded by 20 twitches.
‡ 1½ seconds actual.

The five experiments of Table IV were made by myself. They were followed by the experiments of Table V on 2-second tetani, made independently by Dr. McKeen Cattell.

Table V.

Temperature, 0° C.	20·2	20·2	20·2	20·2	20·2
" Reduced time," seconds at 0° C.	19·6	19·6	19·6	19·6	19·6
Number of preceding twitches	20	10	42	30	21
H/T*l*, 1st 2-second tetanus	0·675	0·705	0·65	0·55	0·64
H/T*l*, 2nd 2-second tetanus	—	—	—	—	—
H/T*l*, 3rd 2-second tetanus	—	—	—	—	—
H/T*l*, extrapolated to rest	0·74	0·74	0·77	0·64	0·70
H/T*l*, twitches	0·079	0·076	0·088	0·079	0·074

Temperature, 0° C.	20·15	20·15	20·15	20·15	20·15
" Reduced time," seconds at 0° C.	19·5	19·5	19·5	19·5	19·5
Number of preceding twitches	27	24	18	19	22
H/T*l*, 1st 2-second tetanus	0·98	0·925	0·61	0·75	0·89
H/T*l*, 2nd 2-second tetanus	—	—	0·51*	0·64*	—
H/T*l*, 3rd 2-second tetanus	—	—	0·47*	—	—
H/T*l*, extrapolated to rest	1·06	1·00	0·67	0·82	0·96
H/T*l*, twitches	0·085	0·091	0·065	0·068	0·075

* Preceded by 10 twitches.

In the next series, also made by Dr. Cattell, no preceding twitches were allowed except the one or two required to ensure that everything was working. In these experiments it was intended to carry out the following procedure: to the first muscle of a pair to give 2-second, 5-second and 2-second tetani in order; to the second muscle 5-second, 2-second and 5-second tetani. See fig. 6. Often, however, one muscle of the pair broke its bone, or pulled the thread from its tendon, so that a complete comparison was not always possible. The results are given in Table VI.

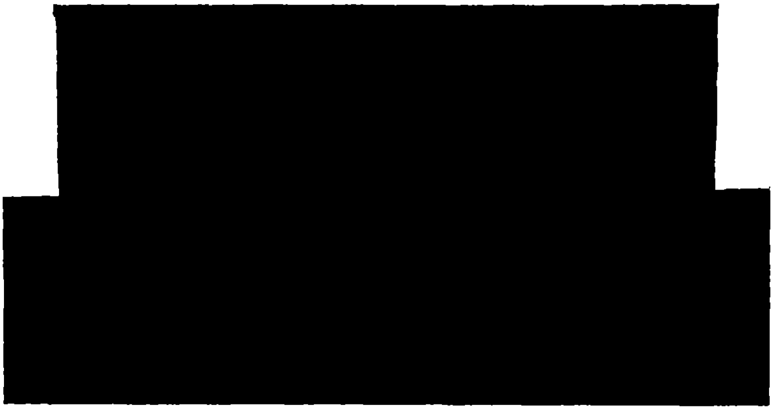

Fig. 6.—Last experiment of Table VI. Tetani of gastrocnemii of Hungarian *R. esc.* at 18·9° C. Top row: 2 seconds, 5 seconds, and 2 seconds; H/T*l* = 0·75, 1·25 and 0·52. Bottom row, opposite muscle: 5 seconds, 2 seconds and 5 seconds; H/T*l* = 1·49, 0·57 and 1·02.

Table VI.

Temperature, 0° C.	20·1	20·1	20·1		19·6
2 second "reduced" to 0° C.	19·4	19·4	19·4		18·3
5 second "reduced" to 0° C.	48·5	48·5	48·5		46·0
H/T*l* (2 seconds)	0·81	0·75	—	0·69	—
H/T*l* (5 seconds)	1·36	1·35	1·35	1·17	1·62
H/T*l* (2 seconds)	0·66	0·61	0·61	0·535	0·72
H/T*l* (5 seconds)	—	—	1·05	—	1·43
H/T*l*, extrapolated to rest (2 sec.)	0·81	0·75	0·73	0·69	0·84
H/T*l*, extrapolated to rest (5 sec.)	1·47	1·46	1·35	1·28	1·62

Temperature, 0° C.	19·6	19·2		19·2	
2 seconds "reduced" to 0° C.	18·3	17·6		17·6	
5 seconds "reduced" to 0° C.	46·0	44·0		44·0	
H/T*l* (2 seconds)	—	—	0·53	—	0·735
H/T*l* (5 seconds)	1·53	1·20	0·98	1·48	1·21
H/T*l* (2 seconds)	0·65	0·535	0·435	0·655	0·55
H/T*l* (5 seconds)	1·25	1·06	—	0·99	—
H/T*l*, extrapolated to rest (2 sec.)	0·77	0·65	0·53	0·77	0·73
H/T*l*, extrapolated to rest (5 sec.)	1·53	1·20	1·09	1·48	1·32

Temperature, 0° C.	19		19	18·9*	
2 seconds "reduced" to 0° C.	17·2		17·2	17	
5 seconds "reduced" to 0° C.	43		43	42·5	
H/T*l* (2 seconds)	—	0·505	0·70	0·75	—
H/T*l* (5 seconds)	1·41	0·83	?	1·25	1·49
H/T*l* (2 seconds)	0·57	0·38	0·72	0·52	0·57
H/T*l* (5 seconds)	0·88	—	—	—	1·02
H/T*l*, extrapolated to rest (2 sec.)	0·69	0·50	0·70	0·75	0·69
H/T*l*, extrapolated to rest (5 sec.)	1·41	0·94	—	1·36	1·49

* See fig. 6.

One fact is immediately evident in Tables IV to VI, viz., the effect of stimulation in lowering H/T*l* in a tetanus. These muscles were unable to recover—they were in nitrogen or air—and the progressive "slowing" due to activity without recovery makes them more economical in maintaining a tension. The same effect was described by Feng (1931, p. 529) for Dutch *R. esc.*, but was very much less than in the present case. In order to compare the present results with one another it is necessary to allow for the effects of previous activity. In some cases successive observations were made on the same muscle, and it was possible to extrapolate to the case of a fresh muscle by assuming—what is approximately true in the neighbourhood of 20° C.—that the heat produced in 20 twitches is the same as that in a 2-second tetanus and one-half that in a 5-second tetanus. In the other cases a mean correction for the previous effect of a 2-second tetanus was calculated as follows:—

(A) *On* H/T*l* *in a 2-second Tetanus.*—(1) As the difference between two successive values of H/T*l* in a 2-second tetanus; and (2) as one-third the difference between two values of H/T*l* in a 2-second tetanus with a 5-second tetanus interposed; these were calculated from 14 experiments and the mean value found to be 0·06.

(B) *On* H/T*l* *in a 5-second Tetanus.*—One-third the difference between two values of H/T*l* in a 5-second tetanus, with a 2-second tetanus interposed, was calculated and found to be 0·11.

These corrections were then applied, and the extrapolated values for a fresh muscle are given in the last row, or rows, of each table.

The experiments of Table VII were performed (by Dr. Cattell) at about 5° C. on 10-second and 25-second tetani of the muscles of frogs previously kept in a cold store at about 3° C.

Table VII.

Temperature, ° C.	4·8		5·3		5·5‡	
10 seconds "reduced" to 0° C.	17·2		18		18·5	
25 seconds "reduced" to 0° C.	42·5		45		46·2	
H/T*l* (10 seconds)	—	0·565	0·67*	—	0·61*	
H/T*l* (25 seconds)	1·10	1·065	1·14	1·04†	1·10	1·01*
H/T*l* (10 seconds)	0·46	0·465	0·53	0·485	0·51	0·41
H/T*l* (25 seconds)	0·98	—	—	0·92	—	0·86
H/T*l*, extrapolated to rest (10 sec.)	0·54	0·56	0·71	0·62	0·65	0·53
H/T*l*, extrapolated to rest (25 sec.)	1·10	1·11	1·22	1·10	1·18	1·05

* Preceded by 18 twitches.
† Preceded by 28 twitches.
‡ See fig. 7.

FIG. 7.—Last experiment of Table VII. Twitches and tetani of gastrocnemius of Hungarian *R. esc.* at 5·5° C. Frog previously kept in cold store at about 3° C. Top row (1) 6 twitches at 3-second intervals, T*l*/H = 12·8; and (2) 10 twitches at 2½-second intervals, T*l*/H = 12·2. Note the spreading out of the twitches and the recovery between the two series. Bottom row; 10-second, 25-second and 10-second tetani, H/T*l* = 0·61, 1·10 and 0·51. Note the low frequency of stimulation, individual twitches initially obvious but gradually disappearing.

The extrapolation was carried out as before, it being assumed that (at 5° C.) 20 twitches have the same effect as a 10-second tetanus and half the effect of a 25-second tetanus.

The results (extrapolated to rest) of all four tables are given in fig. 8. The small circles are for the gastrocnemii, hollow for the high temperatures, full for the low ones. The line drawn through them goes also to the mean point for twitches on the left. In the same diagram is given Feng's mean curve for the sartorii of *R. temp.* (English), and around this are shown by large circles the values found in the present research for the sartorii of *R. esc.* (Hungarian).

Note that for sartorii, the curve and the plotted points are given on twice the scale vertically, to allow for the fact that l in a gastrocnemius is twice the average length of the fibres, or fibre bundles.

It is evident that there is no considerable difference between the sartorii of the frogs used in the present research and those used by Feng. The large circles lie approximately round Feng's curve. It is equally evident, however, that the gastrocnemii fit a very different curve from the sartorii; to make the gastrocnemius curve fit the sartorius one it would be necessary to reduce its time-scale to about 60 per cent. What the explanation of this may be cannot be definitely stated at present; the fact is clear and must be due to some intrinsic property of the muscle fibres. It is possible that a rapid change in the time-scale of the muscle (the "spreading out" described below) during a tetanus is the cause of it.

The dark circles are clearly lower than the light circles, possibly we ought

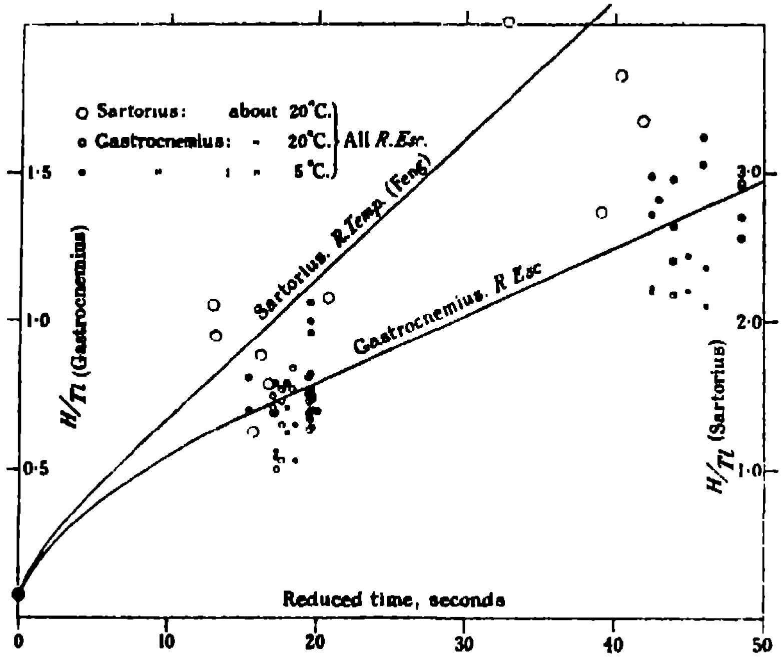

FIG. 8.—H/Tl in isometric tetanic contractions plotted against "reduced time." (The "reduced time" (Feng, 1931) is the actual time multiplied by $(3\cdot1)^{\theta/10}$, where θ° C. is the temperature of the experiment and 3·1 is the temperature coefficient.) The upper curve is copied from Feng's paper, it is for the sartorii of English *R. temp.* The large circles are observations on the sartorii of Hungarian *R. esc.* The lower curve is drawn through the observations on gastrocnemii of Hungarian *R. esc.* Note that the scale for sartorii (right) is twice the scale for gastrocnemii (left), to allow for the fact that the fibres (or fibre bundles) of the latter run on the average only half the length of the muscle. (The value of H/Tl for *t* seconds at any temperature θ° C. can be read off on the curve for $t\,(3\cdot1)^{\theta/10}$ seconds.)

to have employed a rather greater temperature coefficient than the 3·1 which Feng found for the sartorii of *R. temp.* It should be noted, however, that the three frogs used for these experiments at 5° C. had previously been kept in the cold (about 3° C.) and may have been in different condition from the rest.*

The "Slowing" of Muscles as the Result of Activity.—It was shown by Hartree and Hill (1921, *a*, p. 148) that the "efficiency" of a sartorius muscle (*R. temp.*) in maintaining tension during an isometric contraction is increased by previous activity, which is well known to slow its response; they regarded this as an example of the general rule that any factor which makes the twitch slower also makes the tetanus more economical. The relation shown by them (fig. 4), by Feng (1931, fig. 1) and in the present paper (fig. 8), between H/Tl and duration of tetanus, rises initially at a greater rate than it does later on indicating either (*a*) an increase in the ratio of tension to heat (see Hartree and Hill, 1921, *b*, p. 406), or (*b*) a slowing of the muscle in its response to successive elements of the stimulus. Bronk (1930), also employing the sartorii of *R. temp.*, found the economy of maintaining tension $\left(\int \mathrm{T}dt/\mathrm{H}\right)$ to be increased, by increasing the frequency of stimulation, up to the limit of complete fusion, while in a very prolonged contraction, the " economy " might increase more than five-fold, as the result of the slowing associated with the onset of fatigue. Bozler, in similar experiments on the retractor of the pharynx of the snail (1931), found during a prolonged contraction an increase in " economy " of maintaining tension of 6 to 10 times, which was associated as usual with a great slowing of the muscle. With one shock every 3 seconds, at first a very incomplete tetanus was obtained, but at the end a perfectly smooth contraction ; the rate of heat production greatly diminished, in spite of the tension remaining constant. Feng (1931, fig. 2), working with the sartorii of *R. temp.*, found in successive 2-second tetani an increase of Tl/H, which, however, was far less than that observed in the present experiments with the gastrocnemii of Hungarian *R. esc.* In continuous tetanic stimulation he found, as Bronk did, a striking increase in the " economy " of tension maintenance ; this he related to the progressive slowing of the muscle.

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* Other experiments at 5° C. were made with the muscles of frogs not previously kept in the cold, but their response was weak, or their contractions were not well maintained, and the results were of no value.

During the course of the present research striking direct evidence of this slowing was found, which may throw some light on its nature. It was much more obvious, for some reason not yet known, in the muscles of large Hungarian *R. esc.* (at any rate in the batch employed, sent from Hungary at the end of May, used in June and July, and in good condition throughout) than in those of English *R. temp.* or Dutch *R. esc.* In fig. 7 above are shown the twitches and tetani of a gastrocnemius at 5·5° C. The muscle was quite fresh and in excellent condition (twitches about 750 g., tetani about 1,250 g.). The first six twitches, at 3-second intervals, showed a considerable spreading out of the contraction, indeed the sixth twitch lasted almost twice as long as the first. The next 10 twitches (2½-second intervals) showed an even more striking effect, and it is noticeable that the first of this group was just as fast as the first of the preceding series—almost complete recovery in this respect had taken place anaerobically in the 3 minutes between the two series. In the tetani the summation was initially incomplete, but rapidly became nearly complete—an example of the same phenomenon.

The effect is being investigated by Feng and Parkinson, but some further details of it are available. The anaerobic recovery referred to in the speed of contraction is rather rapid, it is nearly complete within half-a-minute of the last stimulus. It is, however, never quite complete; for example, the first twitch in each of the five series of fig. 9 became progressively slower. Moreover, even if the first twitch of a series was only slightly affected by previous activity the spreading out occurred more rapidly in the later than in the earlier series. For example, in fig. 9, the tenth twitch of series (1) lasted about 1·5 times as long as the first; in series (2) 2·0 times; in series (3) 2·2 times; in series (4) 2·3 times; in series (5) 2·7 times.

Fig. 9.—Twitches of gastrocnemius of Hungarian *R, esc.* *First five rows* at 20·2° C. in succession, with several minutes interval, on the same muscle. (1) 10 twitches at 0·9-second intervals, T*l*/H = 13·6, then 2-second tetanus not shown; (2) 20 twitches at 0·64-second intervals (only 13 shown), T*l*/H = 13·7; (3) 20 twitches at 0·64-second intervals (only 13 shown), T*l*/H = 14·2; (4) 20 twitches at 0·6-second intervals (only 16 shown), T*l*/H = 14·6; (5) 20 twitches at 0·6-second intervals (only 15 shown).

Bottom row at 5·3° C., 1st series, 10 twitches, 2½-second intervals; 2nd series, 10 twitches, 3-second intervals; T*l*/H = 11·6 and 12·4.

Note the spreading out, reversed by the intervals between the series.

A striking example is given in the lowest line of fig. 9. Here at 5·3° C. the tenth twitch of the first series at 2½-second intervals was 2½ times as long as the first; the first twitch of the second series was almost identical in speed with that of the first—recovery was nearly complete; the tenth twitch of the second series was again 2½ times as long as the first.

Experiments are being made on muscles poisoned with iodo-acetic acid, in which lactic acid formation and the anaerobic restoration of phosphagen are impossible (Lundsgaard, 1930, p. 65). These may show whether the effects described are due to acid-base changes, or to phosphagen resynthesis.

The phenomenon can be detected, but is not nearly so obvious, in the gastrocnemii of *R. temp.* In fig. 10 the first five rows show the effect as generally found—a noticeable spreading out with short intervals, a barely detectable one with longer intervals. The last five rows show a case in which the spreading out is rather more obvious, but still small compared with that by the Hungarian frogs.

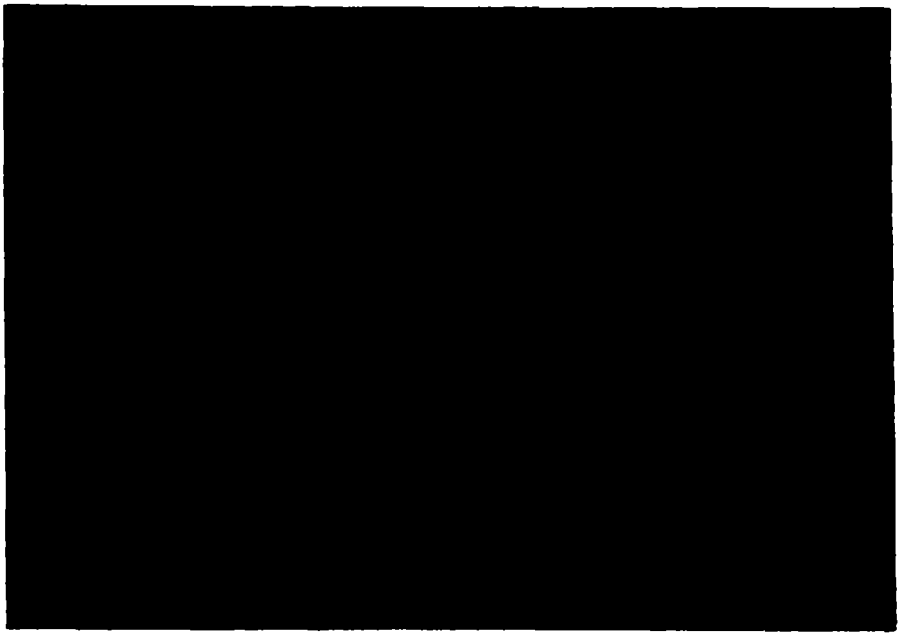

FIG. 10.—Twitches of gastrocnemius of English *R. temp.* Rows 1–5 in succession downwards at 6·1° C., 3-second, 2-second, 1-second, 2-second and 3-second intervals. Rows 6 to 10 in succession downwards at 6·4° C., 3-second, 2-second, 1-second, 2-second and 3-second intervals. Note the much quicker contraction, with less spreading out, than in fig. 9 (bottom row).

Apart from any spreading out it is evident that the gastrocnemii of Hungarian *R. esc.* are considerably slower than those of English *R. temp.* This, together with the much greater spreading out during activity, makes it certain that the former would maintain a prolonged contraction more economically than the latter. This is probably the reason why the gastrocnemius curve of Hungarian *R. esc.* in fig. 8 rises so much more slowly than the sartorius curve of English *R. temp.* It is obviously not wise to compare the " isometric time coefficient "

(Meyerhof) for heat, for lactic acid, for phosphagen or for anything else, of one frog with another, unless we know (*a*) that they have the same "time-scale" of contraction, and (*b*) that the "time-scale" alters to the same degree in both as the result of activity.

In fig. 8 the points for sartorii of the Hungarian frogs, although scattered, are not far from Feng's mean curve for the sartorii of *R. temp.* They are definitely above the mean curve for gastrocnemii. Are gastrocnemii slower than sartorii at the same temperature ? A few attempts to find a difference, by measuring up records made on a fast moving drum, led to no decisive result. There is certainly no considerable difference in speed between the twitches of gastrocnemius and sartorius from the same animal. The sartorii, however, when used for heat measurements, were in oxygen, fully recovered ; in that case, as we have seen already, there will be less spreading out during the early stages of activity. The gastrocnemii had been without oxygen for some little time. It is possible, therefore, that the difference between gastrocnemii and sartorii seen in fig. 8 is due to the greater slowing of the former during the onset of activity, owing to their somewhat less "recovered" condition.

Discussion.

In a recent paper (1931) Lundsgaard has calculated from thermochemical data applied to the lactic acid formed and the phosphagen broken down, the value of T*l*/H in the twitches of a frog's gastrocnemius. For lactic acid he assumed a heat production of 280 cal. per gram. For phosphagen he used two different values (I) 100 cal. and (II) 150 cal. per gram of phosphoric acid set free. For muscles subjected to only moderate stimulation (his Table I) the average calculated value of T*l*/H (after removing the correcting factor 0·69 by which he multiplied *l*) was, according to (I) 10·1 (variation from 8·8 to 12·9), according to (II) 8·3 (variation from 7·5 to 10·3). Both methods of calculation, therefore, give results lower than the mean value for T*l*/H found here, viz. 13·4.

Dr. Lundsgaard informs me that in his experiments the connection between muscle and lever was inextensible, but that he cannot be sure that he worked exactly at the length giving maximum T*l*/H. This, however, could not have reduced T*l*/H from 13·4 to 8·3, and it is doubtful if it could have reduced it to 10·1. Possibly the difference is to be attributed in part to the frogs ; the variations in the present experiments (10·7 to 17·6) can scarcely be due simply to experimental error, and there is some overlap between Lundsgaard's values (8·8 to 12·9 by (I)) and mine.

A more detailed calculation does not appreciably alter Lundsgaard's results. In his Table I (very moderate exercise) the following are average values :—

ΣTl	55000 g. cm. per gram.
Lactic acid formed	0·246 mg. per gram.
Phosphagen broken down	0·602 mg. H_3PO_4 per gram.

According to Meyerhof and Lohmann (1928) the hydrolysis of creatine-phosphoric acid in neutral solution gives 110 to 120 cal. per gram of H_3PO_4; take 115 cal. as the mean. According to Meyerhof (1922, 1930, pp. 207, 221), in cut-up muscles immersed in phosphate solution, or in its enzymatic formation from glycogen and its subsequent neutralisation by phosphate, the liberation of 1 gram of lactic acid is accompanied by the production of 200 cal. To these, however, must be added a small amount of heat due to the acid-base change, which may be calculated as follows.

At p_H 7·0, according to Lipmann and Meyerhof (1930, p. 89) the breakdown of 1 mol. of phosphagen causes a change in the buffer capacity sufficient to neutralise one-third of a mol. of acid without change of p_H. The formation, therefore, of 0·602 mg. = 0·00614 milli-mol. of H_3PO_4 from phosphagen, should allow the neutralisation of 0·00205 milli-mol. = 0·185 mg. of lactic acid, so that only 0·061 mg. remains to be neutralised at the expense of alkali-protein. This is one-quarter of the whole. If the neutralisation of acid by muscle without phosphagen breakdown liberates 9400 cal. per mol. (Stella, 1929) the heat per gram of lactic acid neutralised should be 105 cal.; one-quarter of this is 26 cal., so that the heat per gram of lactic acid in the experiments of Table I should be 226 cal.

The value of Tl/H can then be calculated as follows: $H = 0 \cdot 226 \times 0 \cdot 246 + 0 \cdot 115 \times 0 \cdot 602 = 0 \cdot 125$ cal. = 5350 g. cm. $Tl/H = 55000/5350 = 10 \cdot 3$.

Lundsgaard allowed for the buffer change by taking a larger value for the heat of formation of lactic acid and a smaller one for the heat of breakdown of phosphagen. His mean result, however (10·1) is much the same.

Lundsgaard gives another series of the same kind (Table VIII) which may be treated in the same way :—

ΣTl	105000 g. cm. per gram.
Lactic acid formed	0·57 mg. per gram.
Phosphagen broken down	0·91 mg. H_3PO_4 per gram.

The formation of 0·91 mg. of H_3PO_4 at p_H 7·0 should allow the neutralisation of 0·28 mg. of lactic acid, which is one-half of the whole, so that the heat per gram of lactic acid should be 200 + 105/2 = 253 cal. Thus H = 0·57 × 0·253 + 0·91 × 0·115 = 0·249 cal. = 10600 g. cm. Hence $Tl/H = 9 \cdot 9$.

Thus Tl/H in twitches, when calculated from Lundsgaard's results, is rather less than Tl/H directly observed. It would clearly be of great interest to repeat Lundsgaard's experiments on muscles in which the heat production is measured as well as the chemical changes.

Another series of experiments by Lundsgaard on 2-second tetani (1931, Tables IV and V) again permit the calculation of Tl/H from thermochemical data. His experiments were at room temperature, 18° to 20° C. Feng's reducing factor for 19° C. being 8·6, we may compare Lundsgaard's results with those for a "reduced" time of 17·2 seconds in fig. 8 above; there, for the gastrocnemius, $H/Tl = 0 \cdot 73$, Tl/H therefore 1·37. From the data in Lundsgaard's Table IV (single 2-second tetani) the following mean values may be calculated :—

Tl	5230 g. cm. per gram.
Lactic acid formed	0·176 mg. per gram.
Phosphagen broken down	0·347 mg. H_3PO_4 per gram.

The formation of 0·347 mg. of H_3PO_4 from phosphagen at p_H 7·0 should allow the neutralisation of 0·106 mg. of lactic acid which is 0·6 of the whole, so that the heat of formation and neutralisation of the lactic acid should be 200 + 0·4 × 105 = 242 cal. per gram. From this we may calculate

$$Tl/H = 5230/42650(0 \cdot 176 \times 0 \cdot 242 + 0 \cdot 347 \times 0 \cdot 115) = 1 \cdot 49.$$

This is not far from the 1·37 deduced from the data of fig. 8.

Lundsgaard's Table V gives similar results for the case of three 2-second tetani in succession. The mean values, per 2-second tetanus, may be calculated as follows :—

Tl	5400 g. cm. per gram.
Lactic acid formed	0·214 mg. per gram.
Phosphagen broken down	0·277 mg. H_3PO_4 per gram.

The formation of 0·277 mg. of H_3PO_4 from phosphagen at p_H 7·0 should allow the neutralisation of 0·085 mg. of lactic acid, which is 0·4 of the whole, so that the heat of formation and neutralisation of lactic acid should be 200 + 0·6 × 105 = 263 cal. per gram. From this we may calculate

$$Tl/H = 5400/42650(0 \cdot 214 \times 0 \cdot 263 + 0 \cdot 277 \times 0 \cdot 115) = 1 \cdot 44.$$

This is slightly less than the mean for a single 2-second tetanus, as was to be expected since successive tetani become, as shown above, and by Feng

(1931), progressively more economical. The difference, however, is scarcely significant.

The observed Tl/H in twitches is greater than that calculated; the observed Tl/H in 2-second tetani is less. The discrepancy may imply that Lundsgaard's muscles were slower, and therefore more economical in maintaining a tension, than mine. They were larger (mean 1·7 g. instead of about 1·0 g.), and the muscles of larger animals are usually slower than those of smaller ones. The greater economy in maintaining may have rather more than balanced the smaller efficiency in developing a tension. The heat and the chemical measurements should, however, be made on the same muscles before any final conclusion can be reached.

Summary.

(1) An arrangement is described by which the "initial" heat set free in the contraction of a frog's gastrocnemius can be measured in absolute units. A single fine thermocouple of constantan-iron is employed, the "hot" junction lying inside the muscle. It may be applied to single twitches, to groups of twitches, or to tetani up to 25 seconds in duration.

(2) The mean value of Tl/H, the isometric heat coefficient in a twitch, is 13·4. This is twice the mean value found in sartorii. The difference is due to the fact that in gastrocnemii the fibres, or parallel fibre bundles, run, on the average, only half the length of the muscle.

(3) This explanation of the difference is confirmed by the fact that in twitches Tl/A is twice as great in gastrocnemii as in sartorii, l being the length for maximum tension, T the tension at that length, and A the area of the tension-length curve up to that length. The quantity A/H is the same for gastrocnemii as for sartorii, having the mean value 1·37.

(4) Tl/H increases somewhat in the early stages of activity (anaerobic).

(5) The values of H/Tl in isometric tetani have been measured and plotted against the "reduced" duration of stimulus (Feng). The mean curve is similar to Feng's but lower. This implies that the gastrocnemii of the Hungarian *R. esc.* used in the present research are slower and more economical in maintaining a tension than the sartorii of the English *R. temp.* employed by Feng.

(6) It is well known that an incomplete tetanus, due to a stimulus of low frequency, rapidly becomes more complete as the stimulus is continued, until finally the contraction is quite smooth. In the frogs used (Hungarian *R. esc.*) the same phenomenon is obvious in a series of discrete twitches, the response rapidly spreading out in time. In 15 to 30 seconds' rest (at 20° C.) recovery

occurs (with or without oxygen), so that the first twitch of a new series is nearly as rapid as before. The rate of onset of this "spreading out" considerably affects the value of H/T*l* in a tetanus. Its increase after activity is responsible for the fact that H/T*l* in a tetanus of given duration is less the greater the amount of previous anaerobic activity. The "spreading out" effect is much less obvious in English *R. temp.* than in Hungarian *R. esc.*

(7) The values of T*l*/H in twitches and tetani of frog's gastrocnemii can be calculated from recent experiments by Lundsgaard; if the production of 1 g. of lactic acid from glycogen be assumed to liberate 200 cal., and of 1 g. of phosphoric acid from phosphagen 115 cal., and if allowance be made for acid-base changes, the calculated values of T*l*/H are not far from those observed during the present work.

(8) With special precautions to obtain a high value (working at optimal length, inextensible connections to lever, etc.) the mean value of T*l*/H in twitches of sartorii is about 6·8.

(9) A modification is described of the method recently suggested by Bozler of "calibrating" by condenser discharges. This is far simpler and more accurate than the old method employing an alternating current.

(10) The specific heats of muscle and blood have been determined. For a given water content they are the same. The specific heat can be calculated by assuming a value of 1·0 for the water and a value of 0·32 for the solid.

(11) The density of a tissue depends upon the amount of solid it contains. This provides a simple and accurate method of measuring, without the labour of drying and weighing, the percentage of solid in a muscle.

My sincere thanks are due to Dr. McKeen Cattell of Cornell Medical College, who performed a number of the later experiments for me. They are also due to Mr. A. C. Downing and Mr. J. L. Parkinson for their skilled assistance. The expenses of this research have been borne by a grant from the Foulerton Fund of the Royal Society.

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616.523 — 022.6

Inactivation and Reactivation of the Virus of Herpes.

By J. R. Perdrau, National Institute for Medical Research.

(Communicated by S. R. Douglas, F.R.S.—Received October 15, 1931.)

The experiments, an account of which is given here, were based on two sets of observations—a personal one and one recorded by Zinsser and Seastone (1930). The personal observation relates to the injurious action of air on the virus of herpes. The method in common use for the preservation of this virus consists in the cold storage of pieces of infective brain in glycerol—pure or diluted. If special precautions are taken to exclude air, material so preserved will retain its infectivity for several years. In an ice chest with an average temperature of + 9° C. it has been my experience that unless air is largely excluded (*e.g.*, by a layer of liquid paraffin) the brain will cease to be infective after a period of 3 to 4 months. Under a layer of liquid paraffin and at that temperature the material will still be infective after 2 years. Cold storage at lower temperatures yields better results under both aerobic and anaerobic conditions. The acidity which develops when pieces of fresh tissue are preserved in glycerol has apparently little injurious effect on the virus. The second observation is that of Zinsser and Seastone, who found that a filtrate of herpes virus which possessed a low degree of infectivity and took as much as 11 days to kill a rabbit on cerebral inoculation, recovered its infectivity to such an extent after the addition of cysteine that the incubation period was reduced to 5 days. In a second and more striking experiment an active filtrate of herpes virus lost its infectivity for the rabbit by simple exposure to air for 8 days at room temperature, yet the inactive sample recovered its full infectivity by the addition of cysteine and anaerobic preservation for a further 17 days. Since these observations suggest that exposure to air tends to inactivate the virus of herpes and that the inactivated virus can be reactivated under conditions which favour an increase in the reduction potential, an attempt was made at inactivating broth filtrates of this virus by saturation with oxygen and subsequent reactivation by a process of reduction.

Methods.

The strain used was the one (E.L.I.) isolated from a case of encephalitis lethargica in 1924.

Filtrate.—Only broth filtrates obtained from diffusates were used. The diffusates were prepared as follows. The cerebral hemispheres of a rabbit which had been killed at the height of a typical attack of herpetic encephalitis, were roughly crushed *in situ* with a pair of dissecting forceps and dropped into a tube containing 30 c.c. of Hartley's broth over which a layer of liquid paraffin was floated. The tube was left at room temperature for a few days (5–10) and then was transferred to the cold room until required. Such diffusates will be found active even 18 months later and are easily filtered. After centrifuging the diffusate was filtered through a Pasteur-Chamberland L2 or L3 candle at a negative pressure of 20 cm. Hg. As the filtrate obtained from the first candleful of the diffusate was frequently found to be inactive, it was usually rejected. The rest of the filtrate, unless used immediately, was sealed with vaseline and kept in the cold.

" *Oxidation.*"—A small quantity (about 2 c.c.) of the filtrate was " oxidized " in a test tube by passing a current of oxygen through it with a fine capillary pipette for at least 5 minutes and, as soon as the froth had settled down, the tube was sealed with vaseline and restored to the ice chest until required for use.

Reduction.—The reduction of the " oxidized " sample—with one exception, when cysteine was used—was effected by adding to each cubic centimetre two small drops of a colloidal preparation of palladium black and passing a current of hydrogen. The reduced sample was usually tested immediately after reduction and subsequently sealed with vaseline and kept in the cold.

Animal Tests.—All tests consisted of a cerebral inoculation of 0·2 c.c. into young Dutch rabbits, the passage virus being invariably fatal to rabbits by this route.

Thirteen filtrates were treated as stated above and only four of them were found to be vulnerable to oxygen.

The " oxidized " sample of the first filtrate tested (No. 615) proved fatal on cerebral inoculation after an incubation period of 8 days in each of two experiments. The same " oxidized " samples on subsequent reduction, one with cysteine and the other with palladium and hydrogen, killed in 4 days and 5 days respectively.

Although this result was encouraging, it was hoped that a more complete inactivation might be obtained in view of the striking observation recorded by Zinsser and Seastone, and this was attained with the next filtrate tested (No. 714). In a first experiment, this filtrate in the " oxidized " form took 8 days to kill a rabbit, and 3½ months later the same sample after reduction with palladium and hydrogen killed in 5 days. In a second experiment the "oxidized" sample produced no symptoms of any kind on cerebral inoculation, but one month later after reduction with palladium and hydrogen it killed a rabbit in 4 days.

Filtrate.	Age of filtrate.	p_H.	Oxidized.		Reduced.	
			In cold store with O_2.	Result.	In cold store with O_2.	Result.
615	Fresh	—	5 days	Killed in 8½ days	11 days	Killed in 4 days
615	Fresh	—	3 hours	Killed in 8 days	3 hours	Killed in 5 days
615	7 months	7·1	4 days	Killed in 4 days	—	—
714	Fresh	—	42 hours	Killed in 4 days	—	—
714	1 month	—	3 days	Killed in 8 days	3½ months	Killed in 5 days
714	1 month	—	3 days	Survived	1 month	Killed in 4 days
890	Fresh	7·3	3 days	Survived	13 days	Killed in 6 days
890*	1 month	7·5	25 days	Survived	32 days	Killed in 6 days
890	1 month	7·6	9 days	Killed in 5 days	—	—
890	1 month	6·8	4 days	Survived	14 days	Killed in 5½ days
890	1½ months	7·4	16 days	Survived	28 days	Killed in 7 days
890	1½ months	7·4	4 days	Survived	16 days	Killed in 8 days
890	1½ months	7·5	4 days	Killed in 4 days	—	—
944	Fresh	6·0	4 days	Survived	14 days	Killed in 8 days

* In a first experiment the oxidized sample killed a rabbit, but the reduced one did not. This contradictory result was due to an interchange in the numbers of the animals inoculated, and the mistake was discovered before the result of the experiment was known. A fresh series of inoculations gave the result stated above.

This striking result, of completely inactivating a broth filtrate of herpes virus with oxygen and subsequently restoring its full infectivity to the inactivated sample by a process of reduction, was obtained on six other occasions with two more filtrates. With one filtrate (No. 890) this was done with five different samples over a period of 6 weeks. With yet another filtrate (No. 944) a similar result was obtained once, but the experiment could not be repeated as the stock filtrate rapidly lost its infectivity, which was already somewhat poor, since the incubation period for the reduced sample was as much as 8 days. All the animals which failed to develop any symptoms after inoculation of the "oxidized" samples were subsequently tested for immunity. No evidence was obtained of any immunity having been conferred on these animals, as they all succumbed to the passage virus in the usual incubation period of 4 to 5 days. In the accompanying table only the successful inactivations and reactivations are given. A few experiments were undertaken with a view to overcoming the resistance to oxygen of the other filtrates tested. None of the procedures adopted proved successful and they will be referred to very briefly. An examination of the p_H of the vulnerable filtrates pointed to somewhere around neutrality as the optimum range, yet, when the reactions of the non-vulnerable filtrates were so adjusted by the addition of phosphate, no better results were obtained. On the other hand, a vulnerable filtrate, such as 890, was still oxidizable when the reaction was varied from 6·8 to 7·5.

It was also noticed that the catalase content of the resistant filtrates seemed to be higher than that of the vulnerable ones, but the addition of hydrogen peroxide (Merck's perhydrol), so as just to neutralize the catalase without impairing the infectivity of the filtrates, did not render the latter more vulnerable to oxygen. Hydrogen peroxide in excess of the amount required to neutralize the catalase apparently always killed the virus and, in consequence, subsequent reduction failed to reactivate it.

These experiments, though few in number, are recorded because of the interesting fact which they demonstrate, namely, that the virus of herpes, after being subjected to a process which can only be interpreted as one of oxidation, becomes inert and incapable of infecting or of immunizing a susceptible animal, and is yet alive, and can be restored to full activity by a process of reduction in the test tube. That a similar reactivation of the virus is not effected by the tissues of the living animal after an injection of the inactivated virus is surprising, in view of the high reduction potential of the interior of the living cell. The fact that only 4 out of 13 filtrates were found to be vulnerable to oxygen, although they were all prepared in the same way with the same strain of virus and with the same broth, would seem to indicate that the oxidation is effected by the intervention of a third substance, which is not always present in these filtrates. A similar conclusion is suggested by the observation that one filtrate (No. 615) which was originally partially inactivable, ceased to be so on keeping. It was thought that an enzyme capable of using molecular oxygen might be responsible for the oxidation of the virus, and a small series of experiments was put up with tyrosinase.* Three filtrates, which had resisted the action of oxygen *per se*, were not rendered more vulnerable to oxygen by the addition of tyrosinase.

The effect of oxidation and of reduction on viruses, apart from the observations already quoted about herpes, has been studied principally on the filtrable tumours of birds.

According to Pirie and Holmes (1931), who have recently reviewed the literature and published a fresh series of observations, the inactivation of a

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* I am indebted to Mr. H. W. Dudley for preparing the tyrosinase from meal worms.

Rous filtrate by incubation at 37° C. is due to an oxidative change, since partial reactivation of the filtrate can be effected by suitable reducing agents.

According to Zinsser and Seastone, certain experiments recorded by Berger (1922) and Perdrau (1925) may possibly be explained on lines similar to those considered in this paper. In these experiments fresh brain from fatal cases of encephalitis lethargica proved non-infective to rabbits, but after preservation in glycerol for a few weeks the virus of herpes was recovered from the same material.

A similar observation has since been recorded by Da Fano and Perdrau (1927) and Nicolau (1930) in the chronic herpetic encephalitis of rabbits. There is here more than a bare possibility that the inactivation of the virus in the fresh material may be due to the action of a specific antibody. The relationship between these two sets of observations is now being investigated.

Summary.

Broth filtrates of the virus of herpes can be inactivated by oxygen and subsequently reactivated by a process of reduction. This phenomenon is not a constant one, since it was observed in only 4 out of 13 filtrates, but the experiment could be repeated on filtrates which had once proved vulnerable to oxygen.

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549 . 675 : 541 . 183 . 59

The Sorption Process in the Zeolite Chabazite.

By Meredith Gwynne Evans.

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Abstract.

(1) The changes in the zeolite chabazite during sorption have been investigated by means of X-ray examination of single crystals and of powders.

(2) The heats of sorption for very small additions of ammonia to the zeolite have been measured.

(3) A theory has been advanced to explain the results.

(*The full paper is printed in* 'Proceedings,' A, *vol.* 134, *pp.* 97–102.)

612.79:535.34

Measurements of Transmission of Radiation through a Non-Homogeneous Medium, viz., the Horny Layer of the Human Skin.

By T. C. Angus and H. J. Taylor.

(Communicated by Sir Leonard Hill, F.R.S.—Received June 13, 1931.)

(From the National Institute for Medical Research, London.)

The experiments and methods to be described represent an attempt to answer with greater accuracy a question to which somewhat divergent answers have already been given. The entire work herein described was done with a single sample of clear, dry, desquamated, human skin (horny layer only), taken from the shoulder of a woman. This sample had been detached from the body for some months, and no attempt had been made to keep it in a more humid condition than that which it would naturally attain in the air of the laboratory; but we are indebted to Sir Leonard Hill, at whose instance the work was started, for the opinion that the radiation which penetrates this dead horny layer is the deciding factor in the direct action of radiations on the living cells beneath.

The writers found that two precautions are essential: first, to secure truly monochromatic radiations; secondly, to make allowance for the effects due to the scattering of an optical beam when it is passed through such an uneven medium as a piece of skin. The importance of this last precaution has recently been pointed out by Lucas (1931) and Pearson and Jair (1931).

Purity of Radiations.

Inspection showed that serious errors may be introduced into such work if too much reliance be placed upon the purity of monochromatic light obtained from a single-prism instrument. When light from a powerful mercury vapour lamp was directed into the prism of a monochromator, the lamp being enclosed and the whole experiment being carried out in a darkened room, the whole of the prism was seen to glow with the distinctive colour of the mercury arc, from whichever position the prism was viewed. There was no reason to doubt that some of these visible radiations would be directed into the final lens and slit of the monochromator and so superimposed upon the spectralised radiations which it was desired to measure.

A photographic method was used to measure intensities, and the bluish light we observed would certainly be of sufficient intensity to affect a photographic plate; the errors produced by a scattered bluish light of relatively small intensity mixing with the beam of ultra-violet light to be measured, are apparent when we consider that the horny layer of skin may have a transmission of only 1 per cent. in the ultra-violet, but as much as 50 per cent. in the blue.

To purify our source of light we employed two Hilger quartz monochromators in succession, the last slit of the first instrument being arranged to act as the first slit in the second instrument; with such an arrangement no light could be seen or detected until both the monochromators were set to the same wavelengths, when any given ultra-violet line from the mercury-vapour lamp, which was used as our source, was easily detected and brought to its brightest, by inspection of the fluorescence produced on a piece of uranium glass. The experiments were all made in a darkened room with the stray light from the lamp screened off as much as possible, and the monochromators covered.

Allowance for Scatter.

When a parallel beam of any radiation is passed through a homogeneous medium the fraction transmitted may be found by comparing the incident and transmitted intensities. This is not true for a medium which is not optically homogeneous, for in addition to the reduction in intensity which the beam will suffer on account of absorption in the medium, there will also be a loss of intensity due to scattering in all directions of the once-parallel rays, some of which will fail to reach the measuring instrument, although they may have passed through the absorbing medium.

We employed two methods to overcome this difficulty. (1) The incident intensity was compared with the transmitted intensity immediately the radiation had passed through the medium; the recording surface (a photographic plate) being made so large, and so close to the scattering medium, that there could be little space in which loss by scatter could occur. (2) Completely scattered radiation was used as the source, so that no reduction of intensity due to extra scatter, but only losses by back reflection and absorption could take place when the medium was introduced into the optical system.

Fig. 1 illustrates the first method. The disc of skin, 20 mm. in diameter, is held in a space between two discs of quartz (QQ) 0·6 mm. thick and frosted on both sides. The last of these discs was distant 0·07 inch from the film of an Ilford Special Rapid plate (backed).

The presence and position of these quartz plates was found to be necessary to eliminate traces of "skin pattern" from the final image on the plate, it being impossible to measure a true average density on an uneven image. The advantage of this method is that comparatively short exposures are required, but the disadvantage is that any fluorescent light produced in the skin by the action of the ultra-violet radiations tends to produce an additional darkening of the plate.

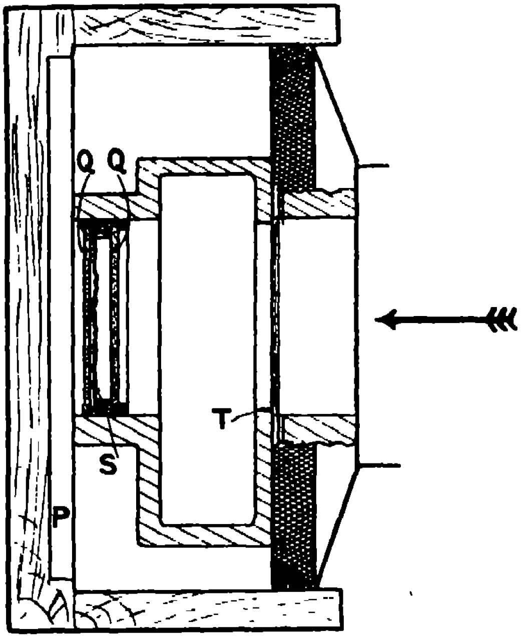

Fig. 1.—P, photographic plate; Q, quartz disc; S, skin sample; T, last slit of monochromator.

In the second method we endeavoured to scatter the incident light so completely that no further loss of light due to scatter could be introduced by the horny layer of skin.

With a more or less parallel beam of light interrupted by the skin sample before it reaches the first slit of the monochromator, a decrease in intensity can be caused by the refraction of some of the light away from the slit, which decrease may be interpreted as a loss by true absorption.

Our method was to scatter the light on its way from the lamp to the first slit so completely that light would arrive at the slit at an equal intensity from every direction. Any additional scatter produced by the skin sample when interposed would not be accompanied by a loss of light reaching the slit; the light merely being rescattered and redistributed so that the general direction of light reaching the slit was the same with or without the skin in position.

Fig. 2 shows how brass rings were constructed to hold three quartz discs. The horny layer of skin was held against one side of the middle disc. Dr. F. C. Toy (to whom we are indebted for much advice) informed us that visible light is only partially scattered by one piece of frosted material; we found that the disc of the sun on a clear day could be viewed through one of our doubly frosted discs, but not through two of them; we concluded that by using three of these discs, separated by spaces of 1·8 mm., we should have light sufficiently scattered for our purpose.

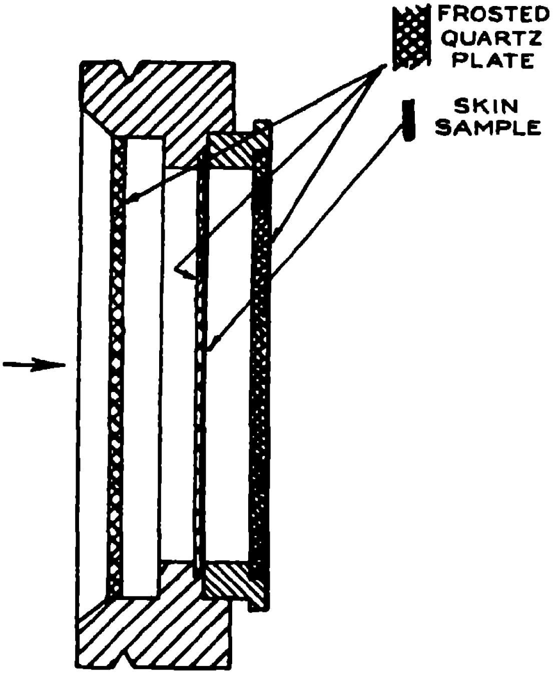

FIG. 2.—Quartz disc holder. Three times actual size.

The advantage of this method is that any fluorescent light from the skin is stopped in the monochromators, while the disadvantage of such an arrangement is that with an intensity so reduced, very long exposures, amounting in some cases to several hours, become necessary. Table I shows the results.

Table I.

Wave-length.	Method 1 (against plate) percentage transmission.	Method 2 (against slit) percentage transmission.
λ 5790	51·3	
λ 5460	54·6	60·5
λ 4359	48·9	
λ 4047	44·5	
λ 3650	43·5	
λ 3341	42·4	40·7
λ 3126	22·2	22·3
λ 3027	6·54	6·25
λ 2925	0·92	0·56
λ 2803	1·56	
λ 2654	0·65	
λ 2537	1·4	1·1
λ 2482	1·7	1·28

One experiment was carried out on wave-length λ 3130, the frosted quartz plates being left out, so that only the direct transmitted radiation was measured, no allowance being made for scattering. The transmission was less than 0·5 per cent. against 22 per cent. shown in the table. This shows the error which may come in if scattering is neglected. In another experiment an exposure of 2½ hours to line λ 2300 was given, with the horny layer of skin against the plate and no use of a scattering medium; in this case there was no image at all on the plate. This line, when the skin sample was not interposed, was clearly visible by fluorescence on uranium glass. Nothing penetrated the skin sample, therefore, in the case of this line.

Fig. 3 shows the curve of percentage transmissions of the horny layer of skin at different wave-lengths of visible and ultra-violet light. These results differ from those of Lucas (1931) who also used the horny layer of the epidermis removed by blistering, in that they show on the average much lower percentage transmissions, and also a much more sudden drop in transmission with decreasing wave-length, which in our case appears to begin at λ 3300 and reach a first minimum at λ 2900, whereas Lucas's curve shows a more gradual drop from λ 4000 to λ 3000 and a very sudden drop from λ 3000 to λ 2900.

The explanation of these results may be that Lucas used some liquids, notably glacial acetic acid, on the skin sample. This would reduce the effect of scattering and back reflection quite considerably. Pearson and Jair (1931) publish a curve which shows a steady fall from λ 4500 to λ 2900 with almost a complete cut-off at λ 2800, and a rise to 5 per cent. at λ 2500; our percentage values are in closer agreement with those of these workers than with those of

Lucas. In general character our curve agrees fairly well with both the recent publications referred to.

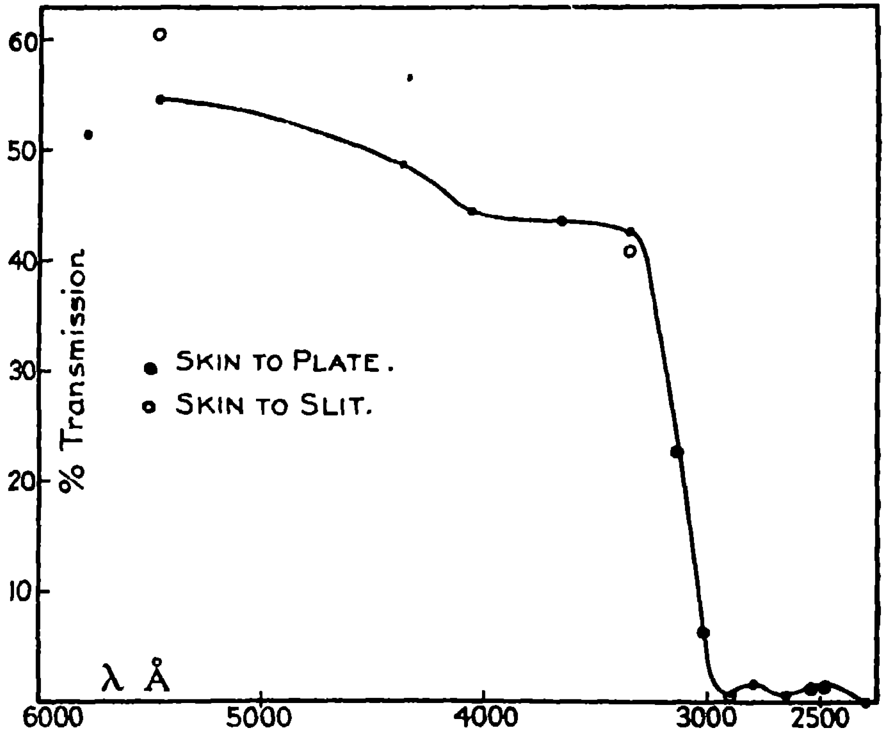

FIG. 3.

Photographic Details.

The comparison between the intensity of the beam of radiation from the lamp through the diffusing media, and the same beam of radiation after having been passed through the diffusing media and the skin, was made by causing these beams to produce images of equal density on the same photographic plate. The intensity of the beam falling on the first diffusing surface and reaching the plate without passing through the skin sample, was reduced by the amount necessary to produce an image of equal darkness after the skin sample had been removed; that is to say, a photograph was taken through the skin sample and the quartz discs with a beam strong enough to produce a readily measurable photographic image, the skin sample was then removed and another photograph was taken on an adjacent part of the plate with the light intensity so reduced that an equal, or very nearly equal, degree of blackness was obtained. The intensity required to produce the latter, compared with the incident intensity when the skin is in position, gives the fractional transmission of the skin.

As Dobson (1926) has pointed out, to make accurate comparisons of intensities by photographic means, it is needful to find places on the same plate where densities are equal. In practice it was found to be nearly impossible to reduce our intensity to exactly the right amount and so produce exactly equal blackening; having made our initial exposure through the skin sample we then made three subsequent exposures of the same time, with no skin sample, judging these intensities so that the middle exposure would give approximately the required blackening.

The maximum density of each patch was then measured by a Dobson (1923) photometer and a curve was plotted connecting density with the logarithm of the relative intensity of the light which had produced it. If the density of the patch which had been photographed through the skin sample fell between the three densities of the subsequent "no skin" exposures, we were able to interpolate this density on the curve, and so deduce, from its logarithm, the intensity of incident light which would have produced a blackening exactly equal to that produced by the beam which had traversed the horny layer of the skin.

Fig. 4 shows one of these curves for λ 2537 horny layer of skin and three quartz discs in front of the monochromators.

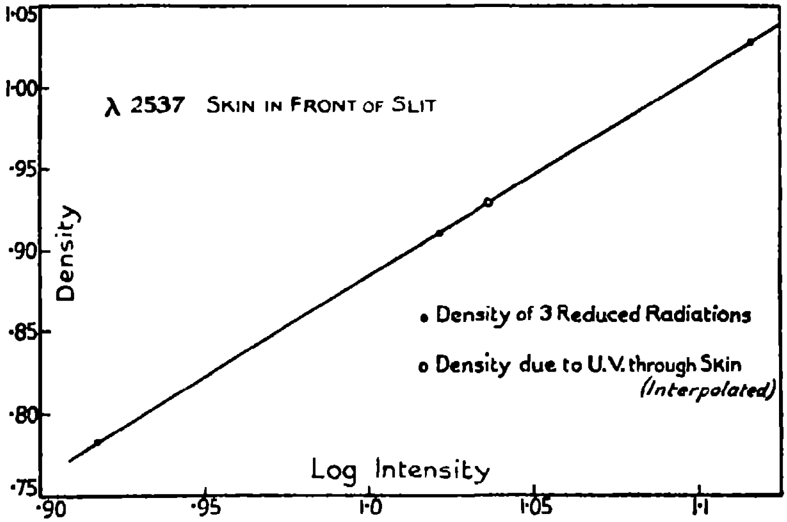

Fig. 4.

Variation of Incident Radiations.

The source of radiations was a Kelvin, Bottomly and Baird mercury-vapour lamp, vertical type, taking 2·1 amperes with a P.D. across the tube of 132 volts. We allowed the lamp to run for not less than 25 minutes before starting an experiment, and we then were able to vary the intensity of the light falling on the two instruments by employing two methods, (1) moving the lamp on its special carriage along a measured scale, (2) reducing the area of the aperture on the lamp.

First Method—Inverse Square Law.—By considering the relative dimensions of the aperture on the lamp and of the slit and the lens distances in and between the first monochromator, we could, on moving the lamp farther away, assume that the intensity of light received on the first diffusing medium, so long as the lamp was at least 40 cm. from the slit, would vary inversely as the square of the distances. We had a scale up to 590 cm. marked out over which the lamp could be moved, giving us a possible reduction of 217 to 1, or of 0·46 per cent. (nearly).

Second Method—Reducing Effective Size of Mercury Arc.—The arc tube of the lamp used was a vertical quartz container of 1½ inches in length. Inspection of the arc through a suitable screen showed that the principal source of visible light was a luminous column or band some 5 mm. wide, with a brightness varying from the middle to either edge. This appearance made us think that the emission of light per unit length of the arc must be very nearly uniform, and induced us to try the arrangement shown in fig. 5.

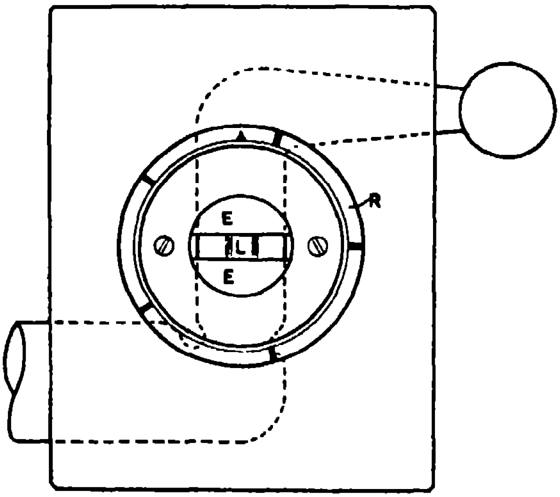

Fig. 5.—Adjustable optical slit. E, edges of horizontal slit; L, luminous Hg arc—exposed length; R, adjusting ring.

An optical slit, somewhat longer than the diameter of the arc tube and variable in width from 0 to 0·35 inch, was fixed in front of, and half-way up, the vertical part of the lamp. It was found that over the limited area disclosible by this variable slit the total intensity was very nearly proportioned to the length of the tube exposed.

An experiment was made to check against each other the accuracy of these two methods. The experiment started with the lamp in its nearest position to the monochromator and the slit nearly closed, a photographic impression was then taken ; the lamp was then moved away in successive steps, but to compensate for the corresponding decreases in intensity the slit in front of the lamp was opened wider each time by an appropriate amount. Equal exposure at each step should then give patches of equal density. Table II shows how nearly these two methods agreed, patches of nearly equal density being obtained with greatly differing distances between lamp to monochromators and with widely different slit openings in front of the lamp.

Table II.

A. Frosted quartz against photographic plate.

Distance lamp-slit.	Aperture (relative width).	Resultant photographic density.
cm.		
40	1	2·640
56·6	2	2·688
80	4	2·683
120	9	2·707
160	16	2·707
200	25	2·718
240	36	2·700

B. -Frosted quartz in front of slit.

Distance lamp-slit.	Aperture (relative width).	Resultant photographic density.
cm.		
56·6	1	0·9850
80	2	1·0480
113·2	4	1·0590
169·8	9	1·0530
126·4	16	1·0400
283	25	1·0510
334·8	35	1·0310

Summary.

(1) A method is described for measuring the percentage transmission of radiations through a non-homogeneous material, viz., the horny layer of the skin.

(2) A series of experiments on this material is described, in which visible and ultra-violet radiations between λ 5790 and λ 2300 were used, pure monochromatic radiations being assured by the use of double monochromators.

(3) Two methods for varying the intensity of the radiations from a mercury vapour lamp by known amounts are described, and a photographic method of measuring the relative intensities of radiations.

This work was carried out at the National Institute for Medical Research with the co-operation of the London Light and Electrical Clinic.

We have pleasure to acknowledge our indebtedness to Sir Leonard Hill, Dr. R. B. Bourdillon, Mr. R. J. C. Jenkins, Dr. Edgar Schuster, and Mr. Smiles, as well as to Dr. F. C. Toy, for much help and advice.

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The Relation between the Pulmonary and Bronchial Vascular Systems.

By J. L. Berry and I. de Burgh Daly.

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I. *Introduction.*

Berry, Brailsford and Daly (1931) showed that perfusion of the bronchial vascular system may be carried out (*a*) by way of the posterior bronchial arteries, or (*b*) in conjunction with the thoracic parietes and œsophagus, or with the œsophagus alone, by way of the aorta. They designated their preparations, lung-thorax-œsophagus, lung-œsophagus, or isolated lung according to the extent of the systemic vascular bed perfused. They also demonstrated that no extensive arterial or venous communications exist between the bronchial and pulmonary vascular systems, the capillaries and possibly the smallest arterioles and venules serving as the only means of direct communication. In the same paper are given in detail the operative procedures carried out prior to the perfusion experiments, together with radiographs and photomicrographs depicting the extent of the bronchial vascular bed perfused. Briefly, the method consists in simultaneous perfusion with pumps of the pulmonary artery and aorta or bronchial arteries, with collection of the blood from the left auricle and azygos vein. It is proposed here to describe the distinctive features of the apparatus and the results of the perfusion experiments.

II. *Method.*

The arrangement used (figs. 1, 2) enables one to measure simultaneously the tidal air, the respiratory pressure ventilating the lungs, the pulmonary arterial and aortic (or bronchial) blood pressures, and the pulmonary arterial, aortic, pulmonary venous and azygos vein blood flows.

The apparatus for perfusion and negative pressure ventilation of the lungs is somewhat different from that used previously for the isolated perfused lungs and heart-lung preparation (Daly, 1927, 1928, 1930). A cast aluminium chamber, A (figs. 1 and 2), fitted with a glass lid to render it air-tight and bored with holes to take the various tubes, takes the place of the glass jar used in the

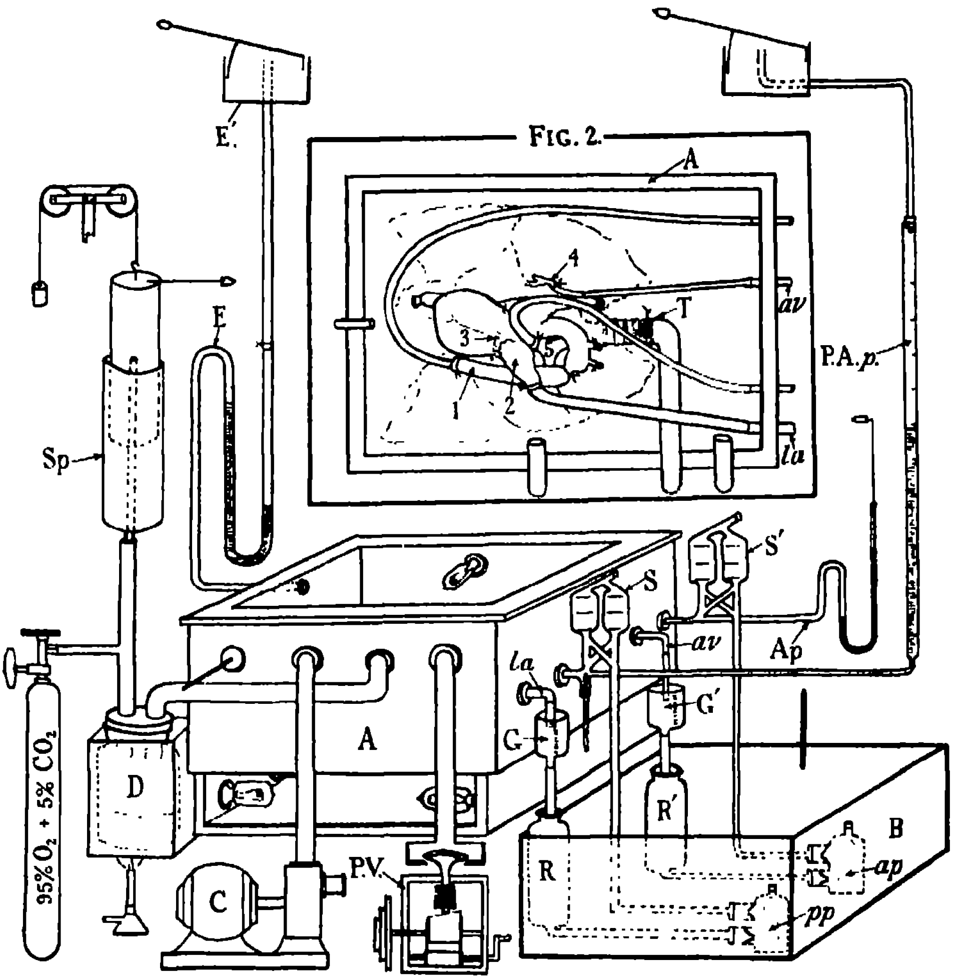

FIG. 2.

FIG. 1.

FIGS. 1 and 2.—Apparatus for simultaneous perfusion of the pulmonary and bronchial vascular systems in lungs under negative pressure ventilation. A = respiratory chamber; Ap = aortic pressure manometer; B = Dale and Schuster pump; C = Cenco exhauster; D = bottle for rendering inspired gas warm and moist; E, E' = manometer and recorder for respiratory pressure; G, G' = graduated cylinders; P.A.*p*. = pulmonary arterial pressure manometer; P.V. = poppet valve; R, R' = venous reservoirs; Sp = spirometer; S, S' = *stromuhrs*; T = trachea; *ap* = aortic pump; *av* = azygos vein outlet; *la* = left auricular outlet; *pp* = pulmonary pump; 1 = thoracic aorta; 2 = left auricle; 3 = clamp at auriculo-ventricular groove; 4 = superior azygos vein; 5 = pulmonary artery.

previous experiments. The descriptions given of similar chambers by Chillingworth and Hopkins (1919, 1920), Liljestrand, van Wijngaarden and Magnus

(1922), Wilson and Hammouda (1928), Drinker and Shaw (1929), and Drinker and McKhann (1929) have been of great help to us in designing the one we use; it measures, internally, 40·7 cm. in length, 30·6 cm. in breadth and 23·0 cm. in depth. Two carbon lamps beneath and one inside serve to maintain the temperature inside the chamber between 34° and 38° C., and water in the bottom of the chamber keeps the air surrounding the preparation moist. The plate-glass top, in contact with a flat rim 2·5 cm. in breadth and sealed with vaseline when in position, is easily removable during an experiment if any adjustments have to be made. Great care is taken to keep the temperatures of the perfusing blood and chamber at approximately the same value.

The double blood-perfusion pump (B) (Dale and Schuster, 1928) in a water-bath is set up with venous reservoirs (R, R′) and with Pavlov and Stolnicov *stromuhrs* (S, S′) on the output sides. For measurement of the venous flow, graduated cylinders (G, G′) are placed above the venous reservoirs, their outlets being fitted with rubber tubing; by pinching the tubing and timing the blood from the left auricular (*la*) and azygos venous (*av*) outlets into the cylinders the rate of flow is obtained. In the earlier experiments the flow was taken by transferring the rubber tubes attached to the bronchial venous and left auricular outlets from the large reservoirs to a graduated cylinder held in the hand, but this procedure produced large errors in the venous flow readings owing to concomitant changes in venous pressure. This will be referred to in a later section. The pulmonary arterial pressure (P.A.*p.*) is registered with a volume recorder in communication with a graduated burette connected to the arterial side of the pulmonary pump, the aortic pressure (Ap) being taken with a mercury manometer.

Rhythmic pressure variations below atmospheric pressure are produced within the chamber by means of a Cenco exhauster (C), which continuously removes air from the chamber, with consequent expansion of the lungs, and by a variable cam poppet-valve (PV) (Daly and Thorpe, 1930) which periodically admits air, so allowing the lungs to collapse. The respiratory depth and the relative durations of the inspiratory and expiratory phases can be altered at will without changing the respiratory frequency.

The trachea (T) is connected to a spirometer (Sp), the air passing over water contained in a large bottle (D) heated to approximately 40° C. The respiratory pressure (intrathoracic pressure) is taken with a water manometer-volume recorder system (E, E′).

The closed air system comprising the intrapulmonary space, the bottle and the spirometer are filled at the beginning of the experiment with 95 per cent. O_2 + 5 per cent. CO_2, which is thus delivered warm and moist to the lungs. Evans (1912) gives the oxygen consumption of the lungs as 0·9 c.c. per gram of heart per hour and the carbon dioxide formation, 0·8 c.c. He found the lungs to be usually from 160 to 200 per cent. of the weight of the heart, so by calculation the oxygen consumption per gram of lung tissue per hour would be 0·5 c.c. and the carbon dioxide formation 0·44 c.c. The lungs of a 10·0 kg. dog weigh approximately 100 g. in our experience, although wide variations from this figure are found in some animals; the oxygen consumption of the lungs, therefore, would be 50 c.c. and carbon dioxide formation 44 c.c. per hour. The approximate total working volume of the closed air space is 2270 c.c. (half the volume of the spirometer = 420 c.c.; bottle + connecting tubes = 1700 c.c.; mean intrapulmonary volume = 150 c.c.; total = 2270 c.c.), and a calculation will show that if the gas therein contains 95 per cent. O_2 + 5 per cent. CO_2 at the beginning of an experiment, it will be 93 per cent. O_2 + 7 per cent. CO_2, 91 per cent. O_2 + 9 per cent. CO_2, and 89 per cent. O_2 + 11 per cent. CO_2 at the end of the first, second and third hour of the perfusion respectively provided no losses take place through the rubber tubing, the visceral pleura and blood in the venous reservoirs. Professor Lovatt Evans informed us he would expect the carbon dioxide losses to be considerable, and on analysis of the gas in the closed system this proved to be so. The cylinder gas contained 95 per cent. O_2 + 5 per cent. CO_2, and in one experiment successive samples of the closed system gas gave 2·7, 3·3, 3·0, 3·1, 2·4 and 2·6 per cent. of CO_2. The initial low carbon dioxide percentage is due to some of the air not being washed out by the gas mixture when the system is filled. We are indebted to Dr. R. G. MacGregor, who took samples at approximately equal intervals of time during an experiment lasting 3 hours. Between the second and third, third and fourth, and the fifth and sixth samples, 135 c.c., 155 c.c., and 225 c.c. respectively of 5 per cent. CO_2 + 95 per cent. O_2 were admitted (total = 515 c.c.) and 150 c.c. of gas mixture were removed for sampling. Since the final volume of the closed air system was equal to the initial volume, 365 c.c. (515–150 c.c.) was lost in 3 hours. From this figure 18 c.c., being the excess O_2 consumption over the CO_2 formed, should be subtracted, making a total loss of 347 c.c. or 116 c.c. of gas mixture per hour. We have not investigated the losses further, but the figures show that the lungs are being respired by approximately 3 per cent. CO_2, which, according to Lohr and Wijngaarden (1924), is on the low side to produce the maximum dilatation of the bronchioles and lung blood vessels.

The blood flows are taken as the average of two or three readings. In those experiments in which the azygos venous flow only amounts to a few centimetres per minute, the graduated vessels, G, G′, are replaced by graduated 10-c.c. cylinders. With still smaller flows the drops are counted. Some mention should be made of the significance of the tidal air and respiratory pressure tracings. The Cenco exhauster and poppet valve are set at the beginning of an experiment so that the chamber pressure oscillates over a range of 10 to 16 cm. of H_2O below atmospheric pressure, care being taken that the expiratory pressure never rises above atmospheric pressure. The respiratory frequency is adjusted in the region of 20 per minute. A resistance to expansion of the lungs, such as is produced by broncho-constriction, is shown by a diminution in tidal air and by an increase in negativity of the inspiratory pressure curve, which results in an augmentation of the respiratory pressure curve as a whole;

very small changes in resistance do not show on the respiratory pressure curve owing to the recorder not being sufficiently sensitive. By this arrangement we have a control on the changes in the tidal air and can say at once whether they are due to an alteration in the lungs or to a variation in the working of the Cenco exhauster. It is evident that the tidal air diminishes if the efficiency of the Cenco exhauster falls, but in this case the respiratory pressure curve also diminishes in amplitude. In order to accommodate the rather large number of recorders, we have used a Brodie-Starling kymograph, fitted with an extension for doubling the recording surface (Daly, 1930, *a*). Means are provided for running wires to stimulating electrodes in the chamber, so that nerves can be stimulated without removing the top of the chamber.

III. *Experimental Observations.*

1. *The Effect upon the Pulmonary Arterial Pressure of raising the Aortic or Bronchial Arterial Pressure.*—Ghoreyeb and Karsner (1913) made a study of the interchange of bloods between the pulmonary and bronchial circulations by simultaneously injecting the two systems with a gelatin-dye mass at various pressures. They concluded that as long as a definite pressure is maintained in either the pulmonary or bronchial circulations, the admixture of bloods is extremely limited. We felt that further light might be thrown upon the problem by observing the changes in blood pressure and flow in one system in response to an artificially produced pressure change in the other, if indeed any response at all took place.

We have observed the effect upon the pulmonary arterial pressure of raising the aortic pressure by increasing the output of the aortic pump, in lung-thorax-œsophagus (L.T.O.) and lung-œsophagus (L.O.) preparations, and of raising the bronchial arterial pressure in the completely isolated lung (L) preparation. The pulmonary arterial pressure response to the systemic arterial pressure rise is somewhat complex; not only are variations found from one animal to another, but the response may change during the course of the experiment. When the aortic pressure is raised for a short time, one of the following changes in the pulmonary arterial pressure may take place :—

A. A simple rise, with return to normal on dropping the aortic pressure (fig. 3, *b*).

B. A simple fall, with return to normal on dropping the aortic pressure (fig. 3, *a*, 4, *b*).

C. A slight rise, with a further transient rise on dropping the aortic pressure (fig. 5).

In any given experiment the pulmonary arterial pressure response gradually diminishes, as shown in fig. 6, where the artificially produced aortic pressure rise at the end of the experiment, although larger than that at the beginning, produces a smaller rise in the pulmonary arterial pressure; moreover, as the

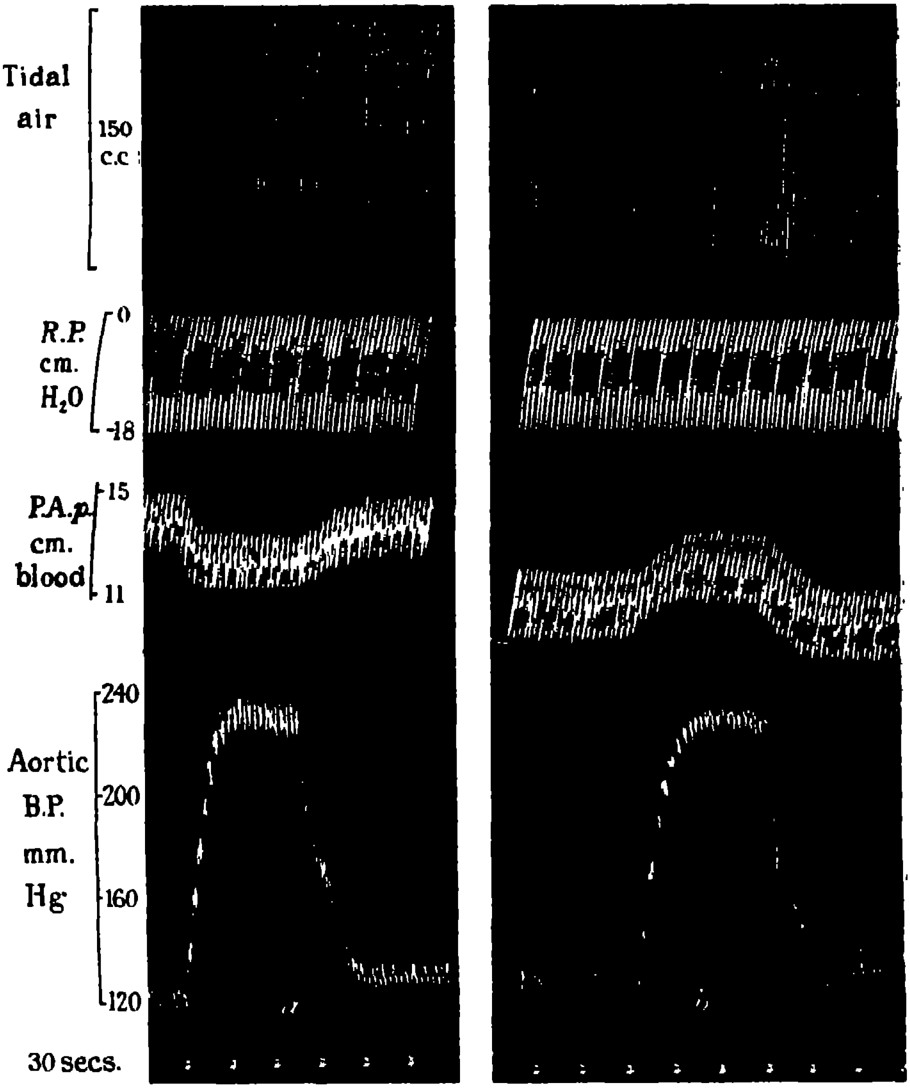

Fig. 3.—Dog. 13·6 kg. Lung-œsophagus preparation. Simultaneous perfusion of pulmonary and bronchial circulations. Effect of raising the aortic pressure. (*a*) Subsequent to 2·0 mg. of eserine injected into the pulmonary artery. (*b*) Subsequent to successive injections of adrenaline 0·2 mg., ergotamine tartrate ("Femergin") 2·5 mg. and adrenaline 0·2 mg. Taken 62 minutes after (*a*).

experiment progresses the pulmonary pressure rise tends to become transient. The fall in pulmonary arterial pressure with constant blood flow as the experiment progresses is usual in lung perfusions with defibrinated blood (Jarisch and van Wijngaarden, 1926).

The response may also change from one type to another during the experiment (fig. 3) or as in one experiment only, be absent early on and appear later.

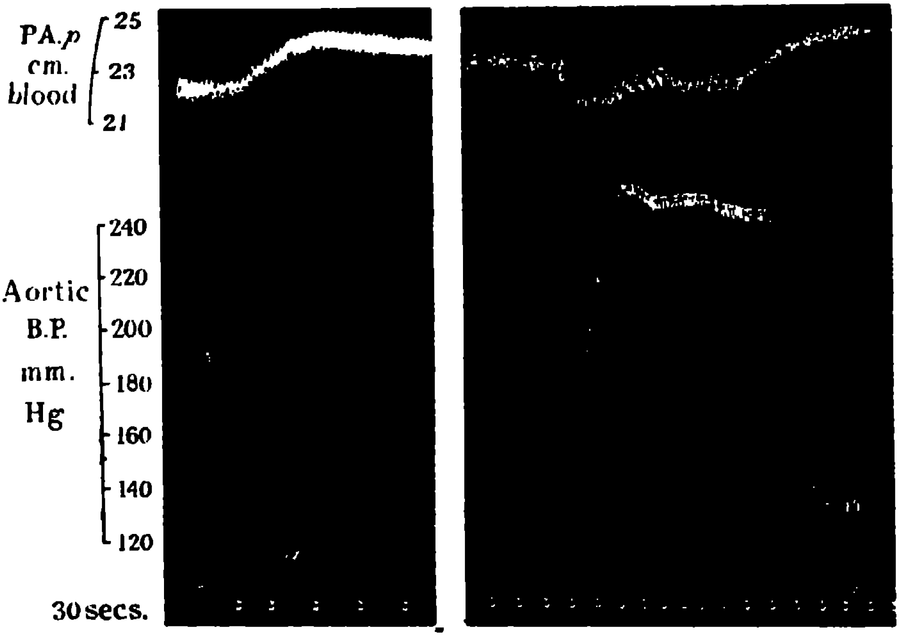

Fig. 4.—Dog. 13·5 kg. Lung-œsophagus preparation. Simultaneous perfusion of aorta and pulmonary artery. (*a*) A fall in aortic pressure causes a rise in pulmonary arterial pressure. (*b*) A rise in aortic pressure causes a fall in pulmonary arterial pressure. The azygos vein cannula was occluded at the signal causing a slight rise in both pressures. Azygos vein flow 11 c.c. per minute.

In fact, we have been unable as yet to correlate the type of response with any definite condition of the preparation or with the experimental procedures adopted. Studies made on 19 dogs gave responses A, B and C in 18, 5 and 3 experiments respectively; thus the more usual effect obtained is the simple rise in pulmonary arterial pressure. Response A occurs equally well before and after atropine or ergotamine tartrate (" Femergin "), and response B and C occur equally well before and after " Femergin," but they have not been tested against atropine. All three types are met with in lung-thorax-œsophagus and lung-œsophagus preparations, but we have as yet only encountered responses A and C in the isolated lung preparations, not response B.

In a few experiments we have found that, although raising the aortic pressure from rather a low to a normal value produces a rise in pulmonary arterial pressure, a further aortic rise causes the pulmonary pressure to fall slightly

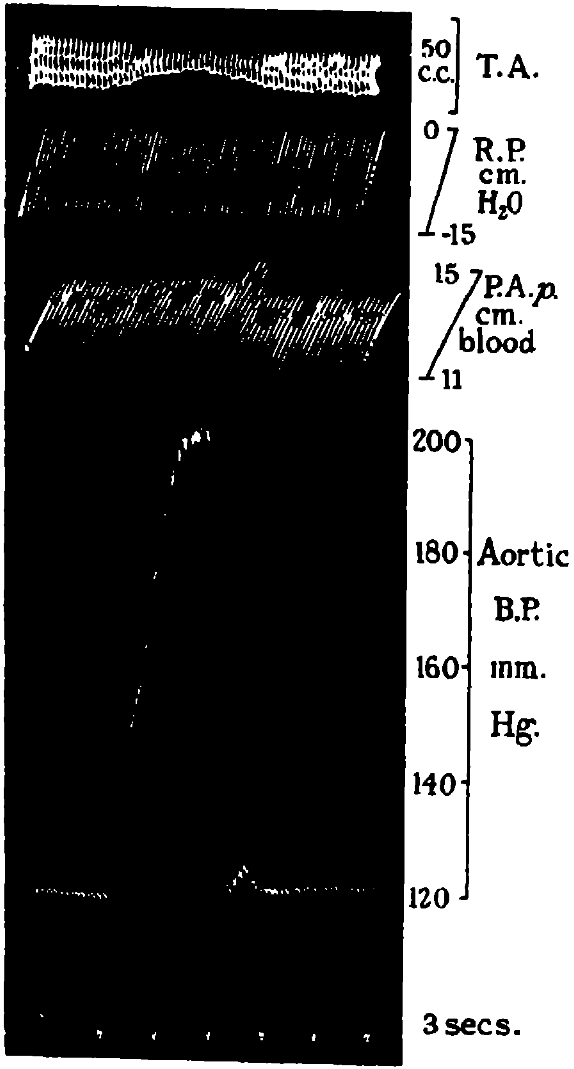

Fig. 5.—Dog. 12·3 kg. Simultaneous perfusion of R. and L. bronchial arteries and the pulmonary artery. A rise in bronchial artery pressure diminishes the tidal air with a slight augmentation of pulmonary arterial pressure (P.A.*p*.) The fall in bronchial B.P. is accompanied by a gradual increase in tidal air and by a transient rise in P.A.*p*.

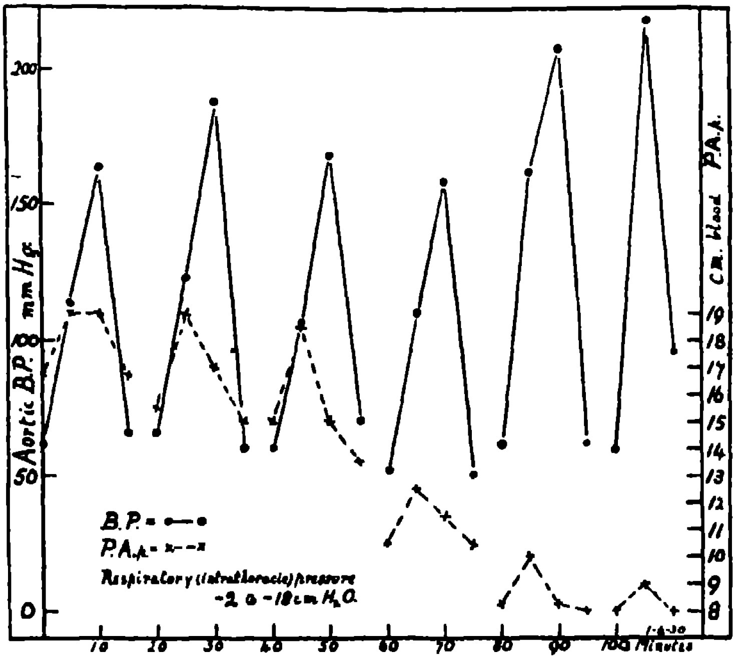

FIG. 6.—Dog. 7·5 kg. Lung-thorax-œsophagus preparation. Effect upon the pulmonary arterial pressure of successive rises in aortic pressure.

(an initial rise may be present), and if then the aortic pressure is brought to its initial value the pulmonary pressure shows a transient rise, followed by a return to its original level (fig. 7). This phenomenon appears to be a mixture of responses A, B and C, and when present can be reproduced.

One other effect of a rise in aortic or bronchial arterial pressure which we obtained in 8 out of 19 experiments should be mentioned, that is, a broncho-constriction. This response, if it appears at all, is generally seen early in the experiment and on only one occasion did it appear late, having been absent previously. We have as yet been unable to satisfy ourselves that this broncho-constriction is due solely to hyperæmia of the bronchial tubes which is sufficient to increase their rigidity, and investigations are being continued in this direction. In this connection, Rindfleisch (1875) states that a rise of blood pressure in the systemic circulation causes static hyperæmia of the bronchial mucous membrane, owing to the anastomosis between the bronchial (systemic) and pulmonary blood vessels.

Being unable to correlate the type of pulmonary arterial response to changes in aortic or bronchial arterial pressure with any of our experimental conditions, we proceeded to measure the blood flow through the pulmonary and systemic circulations, in the hope of obtaining further information.

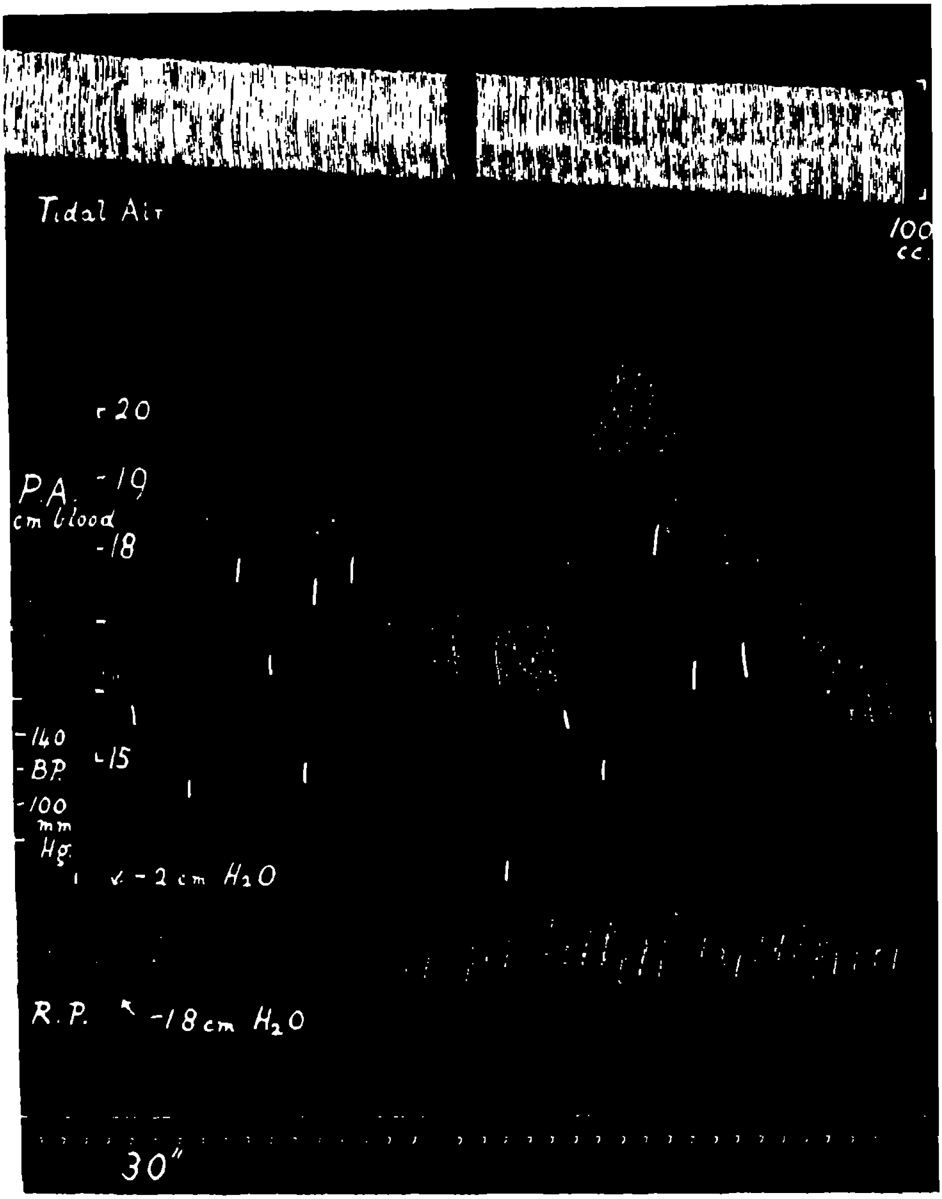

Fig. 7.—Dog. 7·5 kg. Lung-thorax-œsophagus preparation. Effect upon the pulmonary arterial pressure of alterations in the aortic pressure.

2. *Measurements of the Bronchial Blood Flow.*—In measuring the blood flow from the pulmonary veins by a cannula inserted into the left auricle, great care must be taken to keep the pulmonary venous pressure constant while the flow is being taken. We found that transferring the left auricular outflow tube (*la*, fig. 1) from the reservoir to a graduated cylinder held in the hand frequently caused a change in venous pressure with concomitant variations in the pulmonary venous outflow. A rise in the level of the outflow tube of 3 cm. produced in one experiment a diminution in the venous flow of 25 per cent. We therefore fitted to graduated cylinders and measured the flow by pinching their outlet tubes, the time being taken for the blood to fill the cylinder. Laux (1930) drew attention to the fact that with an increasing perfusion pressure the pulmonary inflow is larger than the outflow, and it would appear that this arterial capacity effect which is responsible for the phenomenon finds its counterpart on the venous side of the system. It is clear that failure to eliminate the venous capacity effect during flow measurements will lead to serious errors. Control measurements taken in the manner described above revealed that the sum of the pulmonary and bronchial arterial flows did not differ from the sum of the pulmonary and azygos venous flows by more than 10 per cent. and in general the difference was much smaller than this figure. The main cause of any discrepancy was usually found to be due to small leaks from the systemic arterial vessels. In all three preparations (L.T.O., L.O. and L.), raising the aortic or bronchial arterial pressure with the pulmonary arterial pressure kept constant, increased the pulmonary and azygos venous flows (table), indicating that the bronchial arterial blood drains into the pulmonary veins. Experiment 4 of the table is of particular interest since the posterior bronchial artery of one lung was directly perfused with the pulmonary arterial pressure at zero. An increase in the bronchial arterial pressure from 50 to 210 mm. Hg raised both the pulmonary and azygos venous flows to approximately five times their initial values. The bronchial arterial blood clearly drains into both sides of the heart, but the larger proportion, as judged from experiments 3 to 6 in the table, in which measurements have been taken starting with no pulmonary flow, finds its way into the left auricle. The absolute increase in pulmonary venous flow due to a raised aortic or bronchial arterial pressure does not seem to be affected greatly by the prevailing pulmonary arterial pressure, although the collection of more complete data than we have presented here may reveal some influence.

From experiments 5 and 6 (table p. 330), it would appear that there is also some transfer, although small, of blood from the pulmonary to the bronchial systems

Table.—Simultaneous Perfusion of the Pulmonary and Bronchial Vascular Systems. Effect of changing the Arterial Pressure in one system upon the Blood Flow in the other system.

Experi-ment.	Dog weight, kg.	T.A., c.c.	P.A.p. man. cm. blood.	Cubic centimetres per minute.		Aortic pressure, mm. Hg.	Cubic centimetres per minute.		R.P. cm. H_2O.
				P.A. flow.	L.A. flow.		Aortic flow.	Azygos v. flow.	
1	9·0	90	13·5	223	218	104	14	24·0	0, −11
		90	14·0	223	245	190	183	150·0	0, −11
		87	13·0	223	212	115	14	24·0	0, −11
2	12·0	116	16·0	103	119	138	22	8·0	0, −13
		114	17·0	103	137	230	54	20·0	0, −13
		105	16·5	103	114	144	22	9·0	0, −13
2*a*		105	16·0	396	405	148	23	9·0	0, −13
		89	17·5	396	434	244	50	17·0	0, −13
		89	15·5	396	411	154	24	8·0	0, −13
3	13·0	189	0	0	23	140	—	5·0	0, −14
		189	0	0	41	280	—	8·0	0, −14
		189	0	0	24	144	—	6·0	0, −14
4	9·2	33	0	0	3·5	50	—	1·5	0, −14
		33	0	0	16·0	210	—	7·5	0, −14
		33	0	0	3·0	50	—	1·5	0, −14
5	9·0	—	0	0	7	160	—	1·0	0, −15
		—	33	—	250	160	—	5·0	0, −15
		—	0	0	4	160	—	1·0	0, −15
6	13·0	165	0	0	11	130	—	7	0, −15
		165	22	—	132	130	—	8	0, −15
		165	0	0	10	130	—	7	0, −15

Experiment 1 = Lungs-thorax-œsophagus preparation. Experiments 2, 2*a*, 3, 5 and 6 = Lungs-œsophagus preparation. Experiment 4 = Lung preparation with direct perfusion of the bronchial artery.
T.A. = Tidal air.
P.A.p. man = Pulmonary arterial manometer pressure.
L.A. = Left auricle.
R.P. = Respiratory pressure, expiratory and inspiratory.
— = No measurement made.

since raising the pulmonary arterial pressure, with a constant aortic pressure slightly increases the azygos flow.

Additional evidence that the level of the pulmonary arterial pressure influences the bronchial vascular system is afforded by the fact that a large increase in pulmonary arterial pressure will cause a rise of a few millimetres Hg in the aortic pressure.

We have also found that an increase in the azygos or in the pulmonary venous pressure produces a slight rise in both arterial pressures (figs. 4, *b*, 8). There is no doubt, therefore, that there is a functional anastomosis between the bronchial and pulmonary vascular beds, changes of arterial pressure in the one system altering the arterial pressure in the other, and changes in venous pressure in the one system causing alterations in the arterial pressure of both.

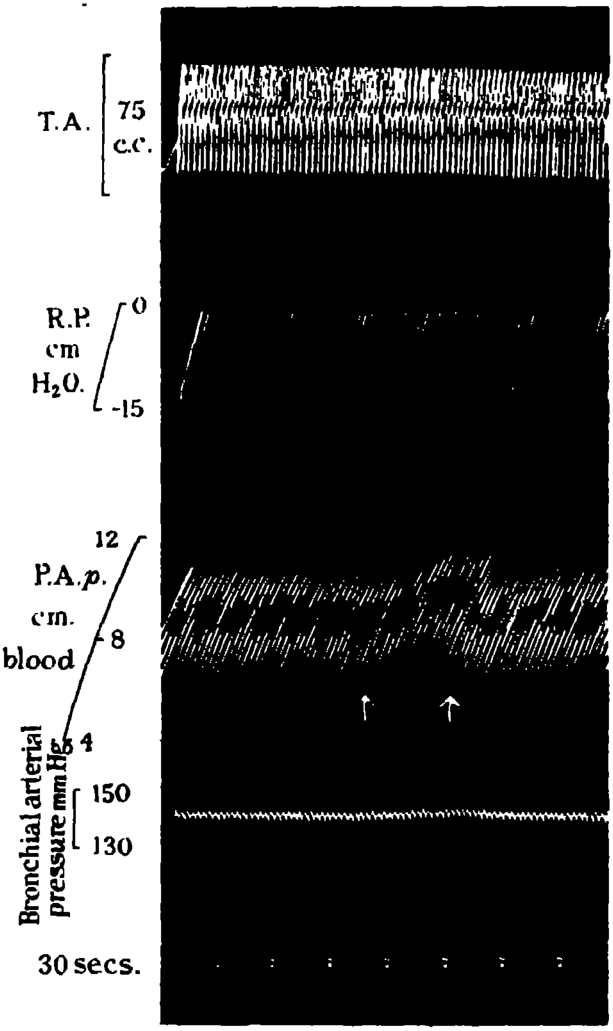

FIG. 8.—Dog. 12·5 kg. Isolated lung preparation. At the first arrow the pulmonary venous pressure was raised 12 cm. blood; at the second arrow, returned to normal.

IV. *Discussion.*

Although Ghoreyeb and Karsner (1913), from the results of their injection experiments of the pulmonary and bronchial circulations, believed that the admixture of bloods was extremely limited as long as a definite pressure is maintained in either circulation, they did think it conceivable that a greater mixture might occur normally. Our perfusion experiments show that with the pulmonary and aortic pressures within normal limits, at least twice as much blood, and sometimes more, passes from the bronchial to the pulmonary circulation as from the bronchial circulation to the azygos veins. How far the bronchial blood invades the pulmonary alveolar capillaries our experiments do not indicate, but the available evidence suggests that under normal conditions the invasion is an extremely small one, the transfer chiefly taking place in the capillary region of the *bronchiolus respiratorius* and possibly the *ductulus alveolaris* (Miller, 1911, 1925, 1925, *a*).

The presence of a vascular shunt pathway from the aorta to the left auricle raises some hæmodynamical problems which are not without interest, especially as the shunt circuit communicates with the capillary bed of the pulmonary circulation. Anrep and Bulatao (1925), using the open-circuit heart-lung preparation, showed that the total output of the right and left heart increases with every rise in aortic resistance, the increase in output being entirely due to the augmentation of coronary flow, which in turn causes a rise in the pulmonary arterial pressure. They obtained no evidence of "back pressure" effects under the conditions of their experiments. Daly (1926), working on the closed-circuit heart-lung preparation, found that with constant ventricular output, an increase in the peripheral resistance produces no alteration, or only a slight rise in the pulmonary arterial pressure, thus confirming in the main the work of Anrep and Bulatao. He thought that if any slight rise in pulmonary pressure occurred it must be due to "back pressure" effects.

Our present observations throw further light upon the problem, for we find in the majority of our experiments that an increase in aortic pressure raises the pulmonary arterial pressure and the pulmonary venous flow in the isolated lungs. Since we keep the pulmonary arterial supply and the pulmonary venous pressure constant, the rise in pulmonary arterial pressure is almost certainly due to the extra amount of blood which we have found passing from the bronchial vascular system into the pulmonary system. The occurrence of the response after full doses of atropine and ergotamine strongly suggests the phenomenon is at any rate in part a mechanical one. This mechanism might be responsible for the slight rise in pulmonary arterial pressure in the closed-circuit heart-lung preparations which sometimes takes place on raising the peripheral resistance even when the ventricular output is constant, and, if this is so, there is now still less reason than before for considering that " back pressure " effects play any part in the phenomenon.

It should be noted that the mechanism will not come into play if the heart-lung preparation is made with the aortic ligature tied immediately distal to the left subclavian artery, for, in this event, the lungs will not receive any blood from the posterior bronchial arteries which give the major supply to the bronchial vascular system (Berry, Brailsford and Daly, 1931). Daly, when making the closed-circuit heart-lung preparations, tied the aortic ligature low down on the descending portion of the arch and, therefore, in some experiments, one or more posterior bronchial arteries would receive blood. Owing to the variability of origin of the posterior bronchial arteries, it is not possible to be certain of the bronchial blood supply to the lungs in heart-lung preparations unless they are examined in every experiment.

One other explanation of the pulmonary arterial pressure rise in response to an aortic pressure increase has attracted our attention, namely, the compression of alveolar capillaries lying adjacent to air tubes by the hyperæmic and more turgid bronchi and bronchioles. We mention it as a possibility, but at the moment have no information to help us.

The fall in pulmonary arterial pressure (response B) caused by an aortic pressure increase may be due to an intrapulmonary nerve reflex, but, being less common than response A, we have not had the opportunity of a full investigation.

The alternative hypothesis, which we have considered, that the increased bronchial blood activity lowers the pulmonary arterial pressure by piezo and " Venturi " effects, is extremely attractive, but further work is needed before a decision can be reached. This hypothesis necessitates the assumption that the broncho-pulmonary anastomotic channels are arranged so as to give rise to piezo-metric and Venturi tube circulations. Swindle (1930) has recently drawn attention to the significance of such mechanical influences in regulating the exchange of body fluids.

Without unduly urging the probability of this type of action being responsible for the effect we have termed response B, we present one tracing (fig. 9) obtained from a perfused system of rubber and glass tubes connected together to represent the pulmonary and bronchial circulations. The arrangement is seen in fig. 10, the anastomotic channels of the two circulations being represented as the side tube, from the narrowest portion of a Venturi tube (V). By adjustment of the pump outputs and outflow pressures, a rise or a fall of perfusion pressure of one pump in response to an increase in flow from the other can be obtained at will. The adjustments are somewhat critical, but the resemblance of their pressure curves to those obtained from the perfused lungs is suggestive. Fig. 9, *a*, was obtained with a low resistance to outflow at P.V., and fig. 9, *b*, with a high resistance to outflow at this point. An increase in number of the Venturi or piezo tubes would bring about a corresponding augmentation in the responses observed, so that the small size of the broncho-pulmonary anastomosis does not militate against our hypothesis.

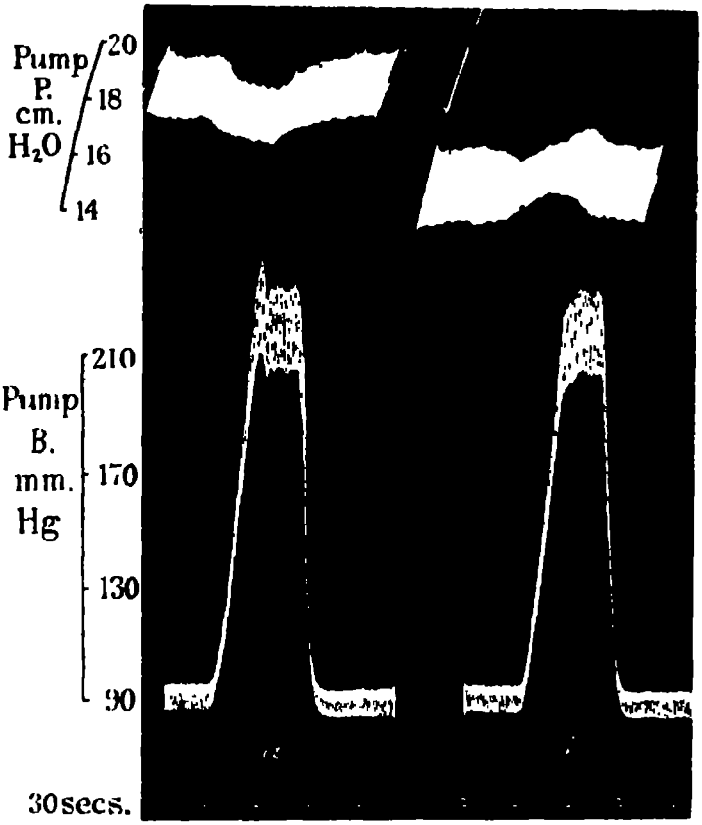

FIG. 9.—Artificial circulation (shown in fig. 10) perfused by two pumps. (*a*) Effect of raising the output of pump B. The clamp on the output tube (P.V., fig. 10) was fully open. Between "*a*" and "*b*" the clamp on the outflow tube P.V. was partly screwed up and the clamp at "*p*" partly released. (*b*) Effect of raising the output of pump B.

At this stage of the inquiry it would be idle to press the analogy further, and for the moment we are content to leave to future experiments to decide as to the truth or falsity of the hypothesis. We would draw attention to the fact that on such a hypothesis the response of the pulmonary arterial pressure to changes in the bronchial arterial pressure might be determined by the pulmonary venous resistance in the preparation.

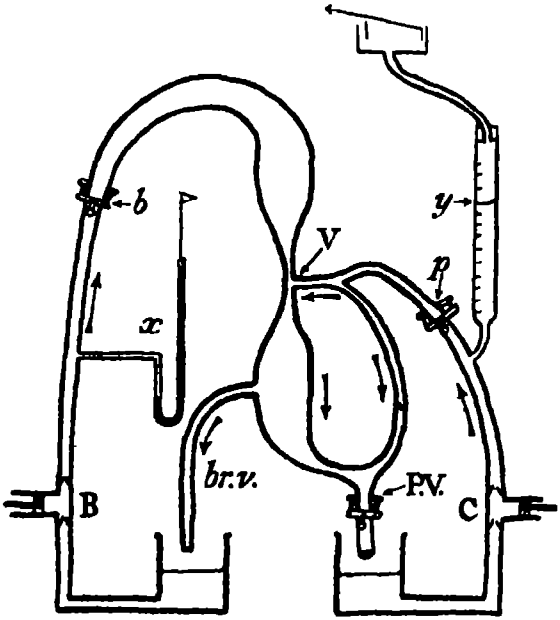

FIG. 10.—Artificial circulation, representing a hypothetical arrangement of the bronchial and pulmonary circulation anastomosis. B = pump supplying the bronchial circulation. C = pump for that portion of the pulmonary circulation which supplies the air tubes. P.V. = pulmonary veins. V = anastomotic branch between pulmonary and bronchial circulations. *br. v.* = bronchial veins. *b* = bronchial arteriole resistance. *p* = pulmonary arteriole resistance. *x* = mercury manometer. *y* = water manometer.

Summary.

(1) A method for simultaneous perfusion of the bronchial and pulmonary vascular systems of the isolated lungs under negative pressure ventilation is described.

(2) Alterations in the bronchial arterial pressure lead to changes in the pulmonary arterial pressure, the pulmonary venous and the azygos venous flows.

(3) There is a functional anastomosis between the bronchial and pulmonary vascular systems.

(4) Hæmodynamical relations between the two systems are discussed.

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The Curariform Action of Quaternary Ammonium Salts.

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The original observation of Crum Brown and Fraser (1869) that metho-salts derived from certain alkaloids did not show the physiological activities typical of their parent bases, but possessed, in common with the simplest quaternary ammonium salt, viz., tetramethylammonium chloride, the property of a curari-like action upon striated muscle, provided one of the earliest examples of uniformity of physiological action among the members of one chemical class. The true curariform nature of the action of quaternary ammonium salts was later confirmed by Rabateau (1873), Brunton and Cash (1884) and other workers. At relatively high concentrations quaternary ammonium salts depress the excitability of muscle to direct stimulation, as was shown by Boehm (1910) and Hober and Waldenberg (1909) for tetramethylammonium and also diminish the conductivity of the nerve (Jodlbaur, 1900), but paralysis to indirect stimulation is produced by these salts at concentrations well below that required to affect either the nerve or the muscle.

Since the original observation of Crum Brown and Fraser, a great variety of quaternary ammonium salts has been shown to possess the curariform action, but few attempts have been made to compare the behaviour of these salts quantitatively under comparable conditions. Kulz (1922) attempted to estimate the minimum concentrations of alkyltrimethyl- and alkyltriethyl-ammonium salts required completely to paralyse the isolated frog's sciatic-gastrocnemius, but he neglected the time factor involved and consequently his results are without quantitative significance. In the alkyltrimethyl series the propyltrimethyl compound was the least active and the higher members of the series up to octyltrimethyl had about the same activity as the tetramethyl compound. The lower members of the alkyltriethyl series were less active than their alkyltrimethyl analogues, but the higher members of both series had approximately the same activity.

It was early noticed that tetraethylammonium salts had a much weaker curariform action than tetramethylammonium salts and that, unlike the latter, they produced an initial increase in excitability accompanied by fibrillary twitching (Brunton and Cash, 1884; Boehm, 1910). Marshall (1914) examined the effect of replacing methyl by ethyl groups in tetramethylammonium chloride, using isolated sartorii and gastrocnemii and found a decrease in activity associated with each successive replacement. Peiser (1920) investigated the action of tetramethyl-, tetraethyl- and tetrapropyl-ammonium salts on the isolated frog's sciatic-gastrocnemius, and confirmed the weaker activity of the tetraethyl compound. He attempted to compare the activities of these three salts at various concentrations by measuring the times required for complete paralysis, but he was unable to obtain reproducible results. The tetrapropyl compound was found to be intermediate in activity between the other two.

Most other attempts to estimate the relative activities of quaternary salts do not call for mention here, as they were based upon experiments with the intact frog, and can have no certain bearing upon experiments with isolated muscle. This paper reports the first part of a systematic attempt to compare quantitatively the action of quaternary ammonium salts on the isolated frog's sartorius, and to discover whether any recognisable relation exists between the structure and physicochemical properties of these compounds and their physiological action.

Technique.

Quaternary ammonium iodides only were used. They were prepared by unambiguous and well-known methods and were carefully purified. An isolated nerve-sartorius preparation from a winter frog (Hungarian) was immersed in Ringer's solution containing a phosphate buffer at p_H 7·2. The nerve was stimulated by a condenser discharge through silver-silver chloride electrodes and the contractions recorded isometrically. When the response of the muscle to indirect stimulation became constant, the buffered Ringer's solution was replaced by a similar solution containing a known concentration of the compound to be tested and the nerve was stimulated at regular intervals of time. The same nerve-muscle preparation could be used for several experiments, the poisoned preparation recovering completely its original response on repeated washing with Ringer's solution.

By plotting the contraction recorded on the drum (expressed as a fraction of the initial contraction) against time, curves such as those illustrated in fig. 1, A were obtained. At relatively high concentrations the curves appear S-shaped, but at lower concentrations a definite latent period is revealed. At high dilutions the curves flatten out and reach an equilibrium position, after which the muscle begins to recover (*vide infra*, fig. 4). Moreover, at high dilutions the curves are not reproducible, probably owing to the long time involved during which the excitability of the muscle changes. Consequently it was found impracticable to measure the minimum concentrations of salts required to effect complete paralysis. Reproducible results, however, were usually obtained with concentrations of salt between 2 and 0·1 millimols/litre for which the times of poisoning were mostly less than an hour. Fig. 1, B illustrates

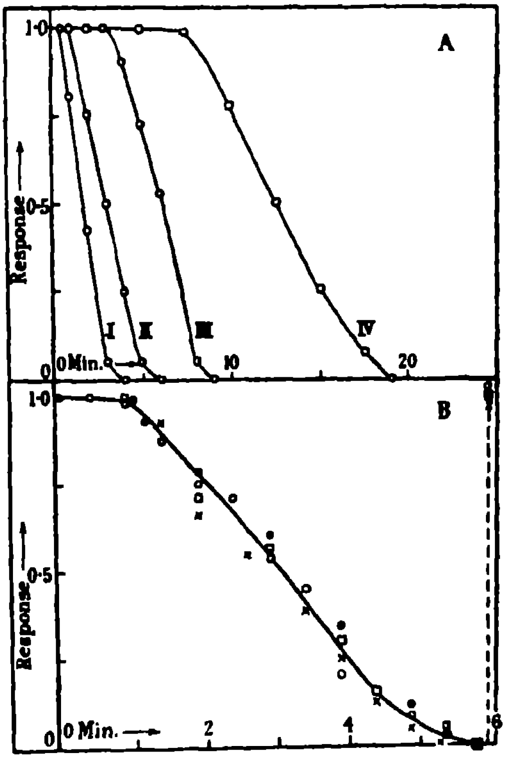

Fig. 1, A.—Nerve-sartorius preparation. Curves I, II, III and IV show the relation between time and the response of the muscle to indirect stimulation (expressed as a fraction of its initial response) when poisoned with tetramethylammonium iodide at concentrations of 2, 1, 0·5 and 0·4 millimols/litre respectively. B.—Nerve-sartorius preparations poisoned with tetramethylammonium iodide (1 millimol/litre). Temperature 14° to 15°. ○ = muscle 40 mm. long; tension of initial response 42 gm.; discharge, 220 volts, 0·005 microfarads and 2 volts, 0·2 microfarads alternatively. ● = same muscle after washing with Ringer and repoisoning; tension of initial response 50 gm., same discharge. × = muscle 35 mm. long; tension of initial response 30 gm.; discharge 2 volts, 0·2 microfarads. □ = muscle 52 mm. long; tension of initial response 48 gm.; discharge 220 volts, 0·005 microfarads. The vertical interrupted line indicates the extent of the response to direct stimulation (induction shock) after complete paralysis.

experiments with tetramethylammonium iodide on three nerve-sartorius preparations in which both high and low tension discharges were employed and in which one preparation, after complete recovery by washing with Ringer's solution, was poisoned a second time. It will be seen that the poisoning curve for a given concentration of this drug is remarkably uniform and reproducible even for muscles differing considerably in size and in tension developed on stimulation. Fig. 1, B also illustrates the completely reversible nature of the action of quaternary ammonium salts, a circumstance which enables more than one drug to be tested on the same preparation. The response of the preparation to high and low tension discharges is shown to be identical at every stage of the poisoning. In routine experiments high tension discharges only were used.

In comparing the activities of different quaternary ammonium salts with widely different molecular weights, it was important to discover whether the rate of diffusion of the drug into the isolated muscle affected appreciably the time of poisoning. This point was investigated by comparing directly the times of poisoning for perfused and isolated muscles. In a number of experiments a gastrocnemius or sartorius muscle was first poisoned by perfusion with buffered Ringer's solution containing a given drug, the rate of poisoning being followed by connecting the distal end of the muscle to an isometric lever and recording the response to indirect stimulation at suitable intervals. Control experiments with trypan red showed that the perfusion fluid reached the muscle within a few seconds. When complete paralysis was achieved, the drug was washed out completely by perfusion with Ringer's solution, the muscle dissected out and poisoned a second time by immersion in the drug. Results for two drugs are illustrated in fig. 2, from which it will be seen that the time of poisoning for a perfused sartorius was not appreciably different from that for the isolated muscle and consequently the time of diffusion of the drug into the muscle must be regarded as negligible in comparison with the time of poisoning. On the other hand, the time of poisoning for an isolated gastrocnemius was much greater than that for the perfused muscle and consequently the time of diffusion into the relatively thick gastrocnemius must be an important factor in the total time of poisoning. These results justify the use of the isolated nerve-sartorius preparation as a standard of comparison of the activities of quaternary salts and tend to invalidate the earlier work of Kulz and of Peiser who used the isolated gastrocnemius.

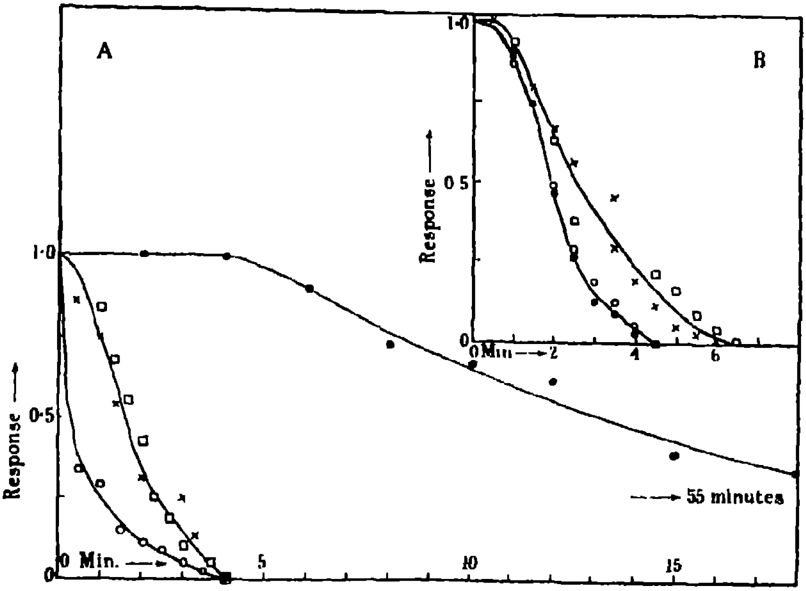

FIG. 2.—Comparison of rates of poisoning for perfused and isolated muscles. A.—2 millimols/litre octyltrimethylammonium iodide—Gastrocnemius: ○, perfused; ●, isolated. Sartorius: ×, perfused; □, isolated. B.—1 millimol/litre heptyltrimethylammonium iodide*—Sartorius: ○, perfused; ●, isolated. 0·5 millimol/litre same drug—Sartorius: ×, perfused; □, isolated.

Results.

The n-*alkyltrimethylammonium Iodides.*—All the members of this series up to the octyl member gave curves closely resembling those illustrated for the tetramethyl compound (fig. 1, A), and the times for complete paralysis for a number of equimolar concentrations are set out in Table I. If these times are taken as a measure of the relative activities of the different members of the series it will be seen that amyl-, hexyl-, heptyl- and octyl-trimethylammonium iodides are approximately equal in activity to tetramethylammonium iodide at concentrations between 2·0 and 0·25 millimols/litre; the propyltrimethyl salt is less active and the ethyltrimethyl compound the least active of the first eight members of the series. In each case the response of the muscle to direct stimulation was unaltered by the action of these salts at or below concentrations of 1 millimol/litre. These results in a measure confirm the cruder estimates of Kulz (1922), except that he found the propyltrimethyl compound the least

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* The temperature during these experiments was slightly higher than that for experiments recorded in Table I, p. 342, and consequently the times of poisoning are shorter.

Table I.

Quaternary salt.	Time in minutes for complete paralysis of isolated nerve-sartorius preparation with *n*-alkyltrimethylammonium iodides at concentrations of				
	2·0 millimols/litre.	1·0 millimol/litre.	0·5 millimol/litre.	0·25 millimol/litre.	0·125 millimol/litre.
Tetramethyl	3·7	5·7	8·7	20	—
Ethyltrimethyl	11	15	45	—	—
Propyltrimethyl	5·5	10	11	65	—
Butyltrimethyl	—	2·5 (7)	6	—	12 (35)
Amyltrimethyl	3·5	5·5	7	10	18
Hexyltrimethyl	4	6	7	10	45
Heptyltrimethyl	—	6·5	—	—	—
Octyltrimethyl	3·7	6	8	10	22
Duodecyltrimethyl	—	30	—	—	—
Phenyltrimethyl	—	5·5	—	—	—
Phenyl benzyl dimethyl	—	5·7	—	—	—

The figures in brackets are times for perfused muscles.

active of the series. Butyltrimethylammonium iodide behaved anomalously. It appeared to be more active than the tetramethyl compound, but it decreased the response of the muscle to direct stimulation at concentrations at which this was unaltered by the latter and the original response of the muscle was not recovered after prolonged washing. In perfusion experiments, however, it appeared to be slightly less active than the tetramethyl compound, also the response to direct stimulation was normal and the muscle recovered completely after perfusion with Ringer's solution. The low activity of duodecyltrimethylammonium iodide is interesting in view of the semi-colloidal properties of this compound. Phenyltrimethyl- and phenylbenzyldimethylammonium iodides have about the same activity as tetramethylammonium iodide at concentrations of 1 millimol/litre.

The Transition Series.—The weak curariform activity of tetraethylammonium salts observed by many previous workers made it interesting to study the effect of successive replacement of methyl or ethyl groups in tetramethylammonium salts. Table II gives the times of poisoning for this transition series. It will be seen that there is a rapid decline in activity in passing from the tetramethyl to the tetraethyl member. Diethyldimethylammonium iodide has a very much weaker action than ethyltrimethyl, while the triethylmethyl and tetraethyl members are about equal in activity and are so weak that neither has any curariform action on the nerve-sartorius preparation at concentrations

Table II.

Quaternary salt.	Time in minutes for complete paralysis of isolated nerve-sartorius preparation with quaternary ammonium iodides at concentrations of		
	10 millimols/litre.	2 millimols/litre.	1 millimol/litre.
Tetramethyl	—	3·7	5·7
Ethyltrimethyl	3·5	11	15
Diethyl dimethyl	34	80	—
Triethyl methyl	255	—	—
Tetraethyl	300	—	—

below 0·5 millimol/litre. At the same time concentrations of triethylmethyl- and tetraethylammonium salts which induce complete paralysis usually reduce the response of the muscle to direct stimulation. These results confirm the earlier work of Marshall (1914), but the actual times of poisoning are considerably different and reveal a much wider divergence in activity among the members of this series than he observed.

The replacement of methyl by ethyl groups in tetramethylammonium leads also to the appearance of new properties. Diethyldimethyl-, triethylmethyl- and tetraethyl-ammonium salts at first increase the response of the muscle to indirect stimulation, an effect which is slight for the diethyldimethyl compound but well-pronounced for the other two. This effect is quite different from the nicotine-like contracture produced by tetramethylammonium salts (Boehm, 1908; Dale and Gasser, 1926) and resembles that produced by guanidine (Peiser, 1920). This action of tetraethylammonium salts closely resembles the effect of increasing the potassium content of Ringer's solution, although it is weaker than the latter (fig. 3). In both cases a preliminary increase in the extent of the response to indirect stimulation occurs and is followed by a fairly rapid decrease and eventual absence of response. Rubidium behaves similarly to potassium, but cæsium has a weaker action and does not show the preliminary increase in response. It is well known that an increase in the potassium content of Ringer's solution leads to the onset of inexcitability of muscle (*cf.* Sereni, 1925), and Dulière and Horton (1929) have suggested that this effect is due to an increase in the concentration of potassium ions in the interspaces between the muscle fibres, which upsets the normal balance of potassium concentration between the interior of the fibres and the interspaces. The preliminary increase of the response to indirect stimulation does not appear to have been noticed previously for potassium.

In addition to the increase in response to indirect stimulation, the triethylmethyl and tetraethyl compounds also produce spontaneous twitching of the muscle.

These results suggest that the action of triethylmethyl and tetraethylammonium salts is not truly carariform in character, but speculation as to its nature would be premature. The subject is being investigated further.

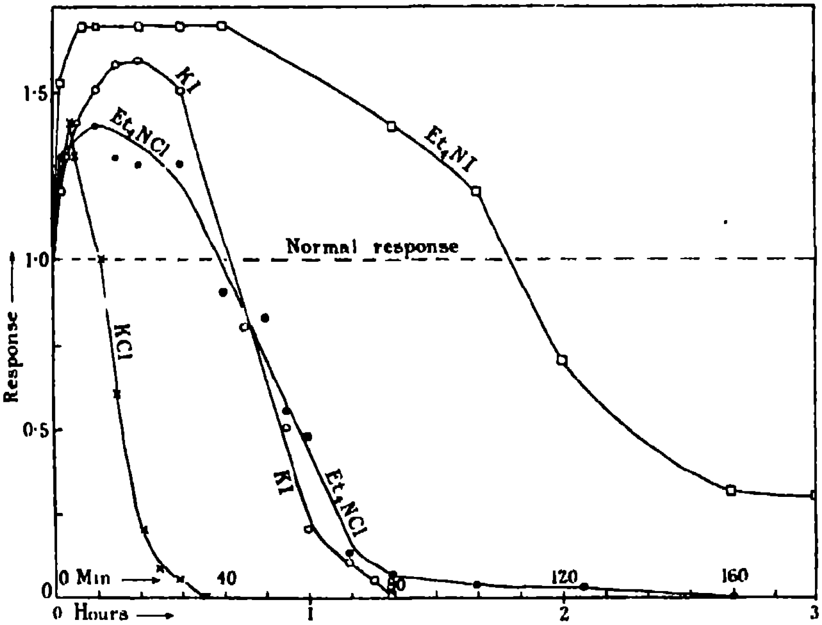

Fig. 3.—Nerve-sartorius preparations. Comparison of the actions of potassium and tetraethylammonium salts at equimolar concentrations (10 millimols/litre) in Ringer's solution containing a phosphate buffer (p_H 7·2).

It is noteworthy that magnesium at fairly high concentrations has a true curariform action (Gunthrie and Ryan, 1910). A solution of magnesium chloride in Ringer's solution containing 10 millimols/litre reduced the response to indirect stimulation of a sartorius muscle to zero in 20 minutes. There was no preliminary excitation, the response to direct stimulation was unaltered and the conductivity of the nerve was not diminished under the experimental conditions used.

Two amine oxides, viz., trimethylamine oxide and methylethylaniline oxide, were examined as their hydrochlorides and found to possess only a feeble curariform action. At a concentration of 1 millimol/litre the latter produced complete paralysis in 200 minutes, whilst the former reduced the response by 86 per cent. in 150 minutes.

Recovery of Muscles Poisoned with Quaternary Ammonium Salts.

In an attempt to measure the minimum concentrations of quaternary salts required to paralyse a nerve-sartorius preparation it was noticed that at high dilutions the response to indirect stimulation eventually reached a minimum value and then began to increase (fig. 4). The recovery of the muscle was not due to exhaustion of the bath as was shown by the fact that the contents could be used to poison a second muscle, whilst renewal of the solution in no way affected the recovery of the muscle (Curve II, fig. 4). This recovery of muscles poisoned with quaternary ammonium salts is remarkable and does not appear to have been recorded previously. According to Overton (1897) and Harvey (1911), quaternary ammonium salts only penetrate living cells extremely slowly, if at all, whereas ammonia and amines do so readily. Curve III (fig. 4) illustrates the rapid recovery of a sartorius poisoned with ammonium chloride. This recovery is probably due to the penetration of ammonia into the cells of the structure upon which the salt acts. Similarly, the recovery of muscles poisoned with quaternary ammonium salts may be due to a much slower penetration of the quaternary ammonium ions into the cells.

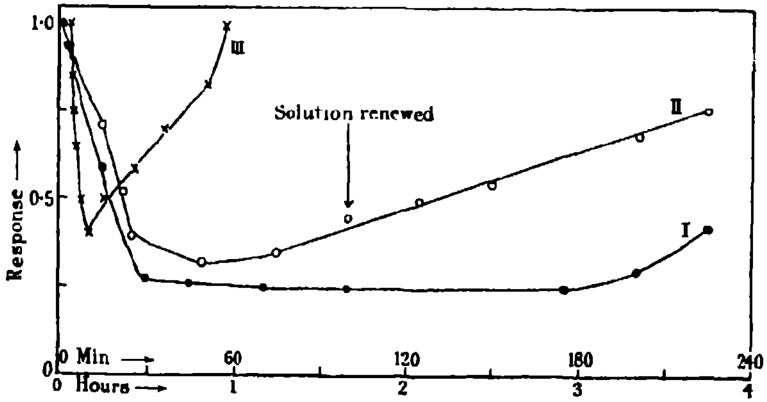

FIG. 4.—Curves I and II show relation between time and response for nerve-sartorius preparations poisoned with 1/75,000 tetramethylammonium iodide (0·07 millimol/litre). Curve III is for ammonium chloride (10 millimols/litre).

Temperature Coefficient.

The temperature coefficient of the action of tetramethylammonium iodide was measured for the temperature ranges 13·5° to 3·5° and 23·5° to 13·5°. The results are shown in Table III. A nerve-sartorius preparation was poisoned at two different temperatures, the muscles being kept in Ringer's solution for half-an-hour at the required temperature before adding the drug. In series A the muscle was poisoned first at the lower temperature, then washed till recovery was complete, brought to the higher temperature and after half-an-hour poisoned again. In series B the muscle was poisoned first at the higher temperature. As the rate of the poisoning process is inversely proportional to the time the temperature coefficient Q_{10} is measured by the ratio of the time at the lower to the time at the higher temperature. The values of Q_{10} obtained were 1·50 for the range 13·5° to 3·5° and 1·54 for the range 23·5° to 13·5°. If the Arrhenius formula

Table III.—Temperature Coefficient for Tetramethylammonium Iodide.

Concentration in millimols/litre.		Time in minutes of poisoning at		Q_{10}.	Time in minutes of poisoning at		Q_{10}.
		3·5°.	13·5°.		13·5°.	23·5°.	
1·0	A	9·25	6·0	1·54	6·25	4·25	1·47
	B	9·5	6·25	1·52	7·0	4·75	1·47
0·5	A	16·5	11·0	1·50	12·25	7·5	1·63
	B	16·5	11·0	1·50	11·0	7·35	1·50
0·33	A	22·0	15·5	1·42	16·0	10·0	1·60
	B	24·0	16·0	1·50	15·5	10·0	1·55

Mean Q_{10} = 1·50. Mean Q_{10} = 1·54.

$$v_2 = v_1 e^{\mu(T_2-T_1)/2T_2T_1}$$

is assumed, where v_1, v_2 are velocities at temperatures T_1, T_2 degrees absolute respectively, μ will have the values 6426 and 7336. These values are rather too high for a purely physical process and suggest that a chemical process is involved in the action of quaternary ammonium salts.

Physicochemical Properties.

Quaternary ammonium salts are strong electrolytes characterised by the great stability of their cations, which closely resemble those of the alkali metals. In this respect they differ from the salts of primary, secondary and tertiary amines, the cations of which readily lose a proton and consequently exist in solution in equilibrium with the free base. One result of this cationic stability is that quaternary ammonium salts penetrate living cells extremely slowly, if at all. The salts of amines are as insoluble in lipoids as quaternary salts, but their ability to penetrate living cells easily is almost certainly due to their hydrolysis to the lipoid-soluble free bases. The slow recovery of muscles poisoned with quaternary salts (*vide supra*) suggests that these salts may in fact penetrate cells slowly.

Stable cationic character appears to be associated with all compounds which possess curariform properties, but as the tetraethylammonium ion shares this property with other quaternary ammonium ions it cannot be regarded as the only essential factor. Walden (1899) gives the conductivities of tetramethyl- and tetraethyl-ammonium iodides at 16 litres dilution as 51·0 and 43·1 respectively, and Ulich (1926) records the ionic mobilities at 25° as 46 and 33 respectively.

The size of quaternary ammonium ions is considerably greater than that of the alkali metals, and consequently the former will probably be less solvated. This view is supported by their relatively high ionic mobilities compared with the alkali metals and also by recent work on the conductivity of their salts in non-aqueous solvents (*cf.* Hartley, 1931). In conformity with the view that quaternary ammonium ions are but little hydrated in aqueous solution, it has now been found that even concentrated solutions fail (1) to salt out a 1 per cent. solution of sodium oleate which is readily salted out by ammonium and alkali metal salts; (2) to precipitate the proteins of ox-serum; and (3) to precipitate a 1 per cent. solution of hæmoglobin. Moreover, the viscosity of ox-serum at 25° was scarcely affected by the addition of tetramethyl-, tetraethyl-, and octyltrimethyl-ammonium iodides in concentrations (100 millimols/litre) well above those used in studying their curariform properties. But even if their small degree of hydration distinguishes quaternary ammonium ions from alkali metal cations, it cannot be used to account for the difference in physiological behaviour of the tetramethyl- and tetraethyl-ammonium ions which are alike in this respect.

It seemed possible that differences in the degree of association or in the extent of complex ion formation of these two ions might be revealed by vapour pressure measurements. The vapour pressures of 0·04 molar solutions of tetramethyl- and tetraethyl-ammonium iodides (1) in water, (2) in phosphate buffered Ringer's solution, and (3) in 0·1 molar glycine were determined by the differential method of A. V. Hill (1930), but in each case differences of less than 1 per cent. were obtained.

Kulz (1922) considered that the curariform activities of the alkyltrimethyl series varied inversely as the solubility of their salts in water. He found with the perchlorates a maximum solubility for the propyltrimethyl member, whilst he obtained minimum physiological activity for the corresponding iodide. It is improbable that solubility in water can have any bearing upon the curariform properties of quaternary ammonium salts. Reason has already been given for regarding the physiological measurements of Kulz as unreliable and with our technique the ethyltrimethyl member was found to be the least active. Moreover, the approximately equal curariform activity of tetramethylammonium, amyl-, hexyl-, heptyl-, octyl- and phenyl-trimethylammonium iodides is not paralleled by their solubilities.

The simple quaternary salts are not appreciably surface active. Even octyltrimethylammonium iodide does not lower the surface tension of water sensibly in normal solution and the salt is only slightly adsorbed on charcoal. The relative adsorbabilities of the cations of the alkyltrimethyl series were estimated by measuring the minimum concentrations required completely to precipitate a 0·1 per cent. arsenious sulphide sol within 2 hours. The amount of ion adsorbed in each case at the minimum concentration was also estimated. The unadsorbed salt was recovered by filtration and evaporation to dryness and its amount measured by diluting its solution in water until the minimum concentration required to precipitate the standard arsenious sulphide sol was again reached. The extent of the dilution enabled the weight of unadsorbed salt to be determined and hence by difference the weight of ion adsorbed. This method provided a ready means of estimating very small amounts of salt once the minimum concentration required to precipitate the standard sol had been determined. The results are shown in Table IV. The relative amounts of different cations adsorbed at the minimum concentrations are approximately constant and equal to 0·1 millimol/gram As_2S_3.

Since constant weights of cation are adsorbed, the minimum concentrations must be in the inverse ratio of the adsorbabilities. Fig. 5 shows the way in which the minimum concentration changes with increase in the number of carbon atoms in the cation, and illustrates the regular increase in adsorbability

Table IV.

Quaternary salt.	Amounts of cation adsorbed expressed in millimols per gram arsenious sulphide.		
	Maximum.	Minimum.	Mean.
Cetyltrimethyl	1·43	1·33	1·38
Duodecyltrimethyl	0·104	1·101	0·102
Octyltrimethyl	0·105	0·073	0·089
Hexyltrimethyl	0·110	0·084	0·092
Amyltrimethyl	0·129	0·021	0·075
Tetraethyl	0·121	0·073	0·098
Butyltrimethyl	0·182	0·099	0·140
Propyltrimethyl	0·310	0·041	0·175

with increase in the weight of the cation. Traube's rule that the ratio of the adsorbabilities of consecutive members of a series is constant is held with fair accuracy for the first eight members of the alkyltrimethyl series. There is no abnormality in the case of ions containing ethyl groups, their adsorbabilities being closely similar to the isomeric ions of the alkyltrimethyl series. With the higher members, duodecyl- and hexadecyl-trimethylammonium iodides,

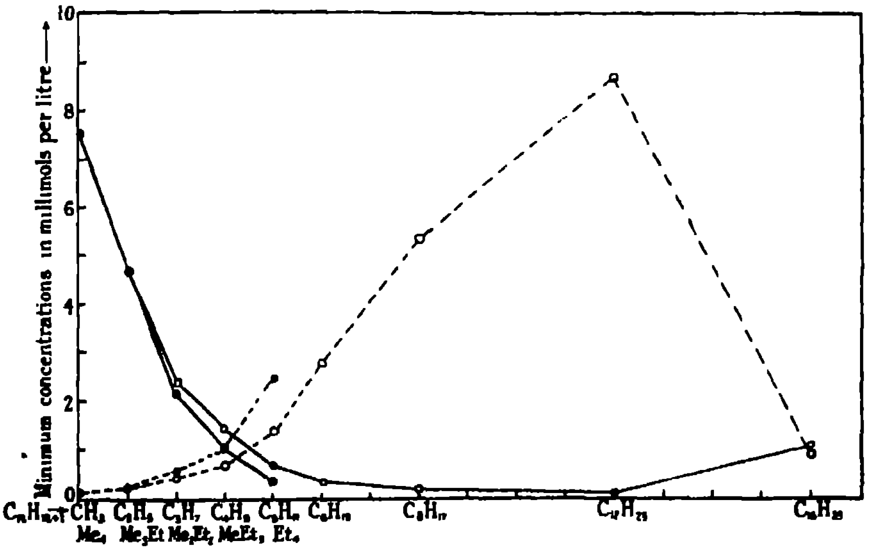

FIG. 5.—Continuous curves illustrate the variation in minimum concentrations of salts $(C_nH_{2n+1})NMe_3I$ (white circles) and $Et_nMe_{4-n}N.I$ (black circles) required to precipitate a standard arsenious sulphide sol with the number of carbon atoms in the cations. Interrupted curves illustrate the relative adsorbabilities of the cations of these two series of salts.

the adsorbabilities are abnormally low, but these salts show semi-colloidal properties and are probably associated. It is evident that the adsorbabilities of the cations of these salts bear no relation to their curariform properties.

Discussion.

The original suggestion of Boehm (1910) that the curariform properties of quaternary ammonium salts is, with a few exceptions, independent of the nature of the radicles attached to nitrogen appears to be largely supported by our results. Salts so diverse in structure as tetramethyl-, octyltrimethyl- and phenylbenzyldimethyl-ammonium iodides were found to have a quantitatively similar activity. An explanation of such uniformity of physiological activity must probably be sought in the common physicochemical properties of these compounds, such as the stability and small hydration of their univalent cations. But among compounds which have several similar properties in common, those relevant to their physiological activity should best be discovered by a study of the properties of exceptional members of the group. Consideration of the physicochemical properties of quaternary ammonium salts has so far failed to reveal what properties either determine the curariform activities of these compounds or account for the exceptional cases.

The low activity of duodecyltrimethylammonium iodide is probably due to its semi-colloidal properties, *e.g.*, owing to association of its ions the concentration of free cations will be less than that in equimolar solutions of other salts. In the case of the amine oxide hydrochlorides the weak curariform activity may conceivably be due to hydrolysis of the salts with production of the lipoid-soluble amine oxides or to reduction to the corresponding tertiary bases which have only a feeble activity.

Compounds containing ethyl groups provide the most striking exceptions to Boehm's generalisation. In the case of the triethylmethyl and tetraethyl compounds the difference is not only one of degree but also of kind, for the preliminary increase of the response to indirect stimulation and the fibrillary twitchings do not occur with tetramethylammonium and salts similar to it in curariform activity. The resemblance between the action of tetraethylammonium salts and the effect of increasing the potassium content of Ringer's solution is also interesting and may repay further investigation. It is possible that the action of the triethylmethyl and tetraethyl compounds is due to a mechanism quite different from that of other quaternary salts or to the superposition of some different mechanism upon a very weak curariform activity. It is well known that tetramethyl and tetraethylammonium salts differ widely in several other of their physiological actions. Thus Burn and Dale (1914) found that the latter exhibited neither the muscarine-like action nor the nicotine-like stimulation of the sympathetic ganglion cells shown by the former, although it exhibited the secondary paralytic phase of the nicotine-like action. Boehm (1908) and Marshall (1914) found that tetraethylammonium salts do not exhibit the nicotine-like contracture of amphibian muscle which is shown by the tetramethylammonium salts, and a similar result was obtained by Dale and Gasser (1926) for mammalian muscle after degeneration of the nerve. But even if the mechanism of the tetraethyl compound is different in kind, the question still remains why it exhibits no curariform action at dilutions at which the tetramethyl compound produces a rapid action.

The mode of action of quaternary ammonium salts is quite unknown. Langley (1906) concluded from his experiments on the antagonism of curari by nicotine that curari acted upon some intermediate receptor—the myoneural junction—between the nerve and muscle, and it is probable that the quaternary ammonium salts act similarly. As with curari, there is a preliminary rapid binding of the salt by the muscle followed by a slower reaction, for if the muscle be immersed in Ringer's solution containing the drug for half-a-minute and be then transferred to Ringer's solution, the paralysis of the muscle continues and is complete in the same time as that when the muscle is left in the solution of the drug during the whole time of poisoning. The preliminary binding is probably due to a rapid diffusion process of the quaternary ammonium ions into the muscle, although it is not clear why they should be so firmly held as not to diffuse out equally rapidly when the muscle is washed with Ringer's solution. This preliminary binding probably corresponds to the latent period revealed with more dilute solutions of drug in the poisoning curves (fig. 1, A). The second slower process probably involves a chemical reaction since the temperature coefficient of 1·5 for tetramethylammonium iodide is higher than would be expected for a purely physical process. This view is supported by the work of Laidlaw (1912) who found a large difference in activity between the dextro and lævo forms of both α- and β-canadine methochlorides in which the optical activity is associated with both carbon and nitrogen atoms. It is difficult to escape the conclusion that the physiological action of optical enantiomorphs involves a chemical reaction when their activities are unequal.

The nature of the chemical reaction postulated remains a matter for speculation. On general chemical grounds the possibilities of reaction for quaternary ammonium salts appear to be rather limited. Any decomposition of the drug is made improbable by the complete reversibility of the physiological action and by the extent to which this action is independent of the nature of the radicles attached to nitrogen. Some permutit-like exchange of ions in which the quaternary ammonium ions replace inorganic cations in the structure upon which the drug acts is a possibility. The value 1·5 for Q_{10}, although too high for a purely physical process is rather lower than that for most chemical reactions, but there is reason to think that reactions of the permutit type have low temperature coefficients. Whitehorn (1923) found Q_{10} equal to 1·6 for the reaction between ammonium chloride and a sodium zeolite. An ionic exchange reaction of the type envisaged would be reversible and would probably depend upon the physical properties of quaternary ammonium ions. It would be difficult to obtain direct experimental evidence of such an ionic exchange, if it occurred, owing to the very small changes in ionic concentrations involved, but it is proposed to explore further the possibilities of such an hypothesis of the pharmacological action of quaternary ammonium ions.

Summary.

(1) The curariform action of fourteen quaternary ammonium iodides on the isolated nerve-sartorius preparation of winter frogs has been studied.

(2) The time of diffusion of the drug into the isolated sartorius muscle has been shown to be negligible compared with the time required to produce complete paralysis, but with isolated gastrocnemii the time of diffusion is not negligible.

(3) In the *n*-alkyltrimethyl series, amyl-, hexyl-, heptyl-, and octyltrimethylammonium iodides are about equal in activity to tetramethylammonium iodide. Propyltrimethyl is less active and ethyltrimethyl the least active of the first eight members of the series. Duodecyltrimethylammonium iodide is less active than the ethyltrimethyl compound.

(4) Phenyltrimethyl- and phenylbenzyldimethylammonium iodides are about equal in activity to tetramethylammonium iodide.

(5) The replacement of methyl by ethyl groups in the tetramethylammonium ion leads to a rapid decline in activity. The triethylmethyl and tetraethyl compounds exhibit an initial increase in the response to indirect stimulation.

(6) Two amine oxide salts were examined and found to have a feeble curariform activity.

(7) Sartorii were found to recover slowly from the paralysis induced by very dilute solutions of tetramethylammonium iodide when still immersed in the solution of the drug.

(8) The temperature coefficient Q_{10} of the curariform action of tetramethylammonium iodide was found to be 1·5 for the temperature ranges 23·5° to 13·5° and 13·5° to 3·5°.

(9) The relative adsorbabilities of the alkyltrimethylammonium ions were found to increase regularly with the number of carbon atoms up to the octyl member and there was no abnormality in the case of the ions containing one or more ethyl groups.

It is a pleasure to record our indebtedness to Dr. F. R. Winton for his helpful criticism and continued interest in this work and to Professor C. Lovatt Evans for his criticism of the manuscript. We are also indebted to the Medical Research Council for a personal grant to one of us (W.M.W.) and to the Chemical Society for grants which have partly defrayed the cost of materials.

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The Extent of Air in the Tracheoles of some Terrestrial Insects.

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Recently a theory was put forward (Wigglesworth, 1930) according to which the extent of air in the tracheal capillaries of insects is determined by the osmotic pressure of the tissue fluids in which these capillaries are bathed. Thus, whereas in a state of rest the terminal region of the tracheoles contains fluid, when the tissues are active the consequent increase in osmotic pressure leads to the removal of this fluid and the extension of air into the finest branches. According to this theory the removal of fluid would thus have a certain physiological significance in improving the supply of oxygen to the active tissues.

These conclusions were based almost solely on experiments on the larva of the mosquito; and in a later paper (Wigglesworth, 1931, *a*), although it was recognised that osmotic pressure is probably in all cases the force which keeps the tracheæ more or less filled with air, it was suggested that " in terrestrial insects, with a limited supply of water, the osmotic pressure of the tissue fluids may, at times, be far higher than in an aquatic insect like the mosquito larva; and this high osmotic pressure might be expected to draw the fluid into such fine tracheoles that any further increase due to muscular contraction will have no significant effect."

It is the object of the present paper to examine this point.

Tracheal Endings in the Flea.

The tracheal endings can be clearly seen during life in comparatively few terrestrial insects, but the adult flea (*Ceratophyllus fasciatus*) is a particularly favourable object for this purpose.

If the flea is held beneath a coverslip and examined, by transmitted light, with the one-sixth inch objective, it can be seen that the air in the tracheoles of the abdomen ends abruptly at a point where the tubes are still comparatively large. In the case of the muscles of the legs and head the air almost invariably extends into the finest branches. This is doubtless due to the activity of these muscles.

If the insect is flooded with water it immediately becomes extremely active, and in a few seconds the air extends into the finest branches in all parts of the body. On removal of the water and admission of air, the fluid slowly rises again in the tubes until it has more or less regained the point from which it started. These effects are precisely similar to those already described in the mosquito larva.

If now the flea is kept beneath the coverslip and the extent of air in a given tracheal branch noted each day (fig. 1), it is found that there is no marked change in the extent of the air on successive days.

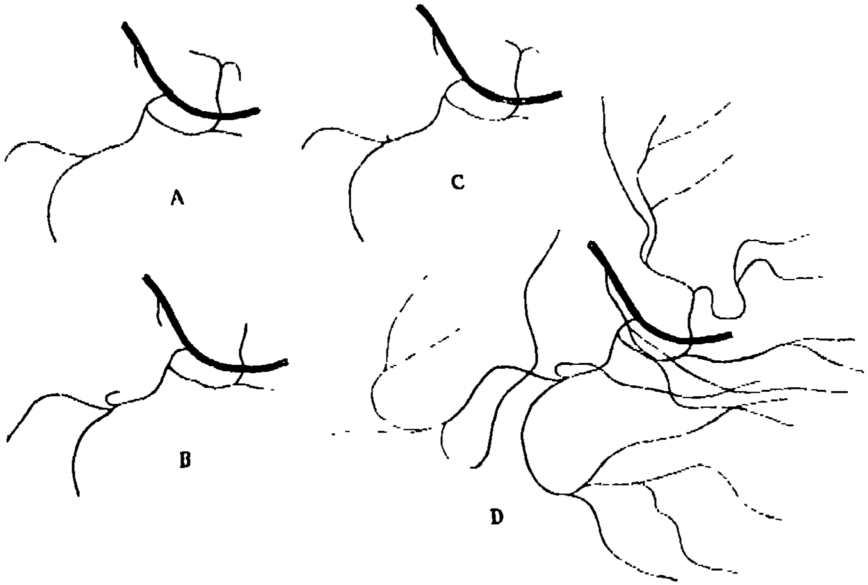

FIG. 1.—Tracheal ending in abdomen of flea, kept under coverslip without feeding. A, May 1, 1931; B, May 3, 1931; C, May 5, 1931, heart no longer beating, flea apparently dead; D, May 6, 1931, flea shrunken and dried. (The tracheoles are visible as black threads when they contain air, invisible when they contain fluid.)

The fleas survived, under the conditions employed, for about 4 to 8 days. Very often the clear fluid in the rectum was evacuated the day before death and the flea then assumed a shrunken appearance; but it was not until some time *after* death that the fluid was absorbed from the tracheoles and the finest branches became filled with air (fig. 1, D).

Thus it would appear that the osmotic pressure is not increased by exposing the insect to dry conditions, so long as it remains alive.

Tracheal Endings in the Mealworm.

The larva of the mealworm (*Tenebrio molitor*) is a particularly interesting insect to study in this connection on account of its prolonged resistance to starvation and drying.

The tracheal endings can be readily seen in small living larvæ (about half-an-inch in length) held beneath a coverslip. The tracheæ in the abdominal segments fall roughly into two groups; a superficial group supplying the muscles of the body wall, and a deep group ramifying over the surface of the gut.

In a larva freshly removed from the food, the superficial branches usually contain air as far as they can be resolved, but in the deeper branches the endings contain fluid. When the larva is held beneath the coverslip, however, and the muscles of the body wall are obliged to rest, fluid gradually appears in the endings of the superficial branches also, though it usually does not rise so high in these as in the branches on the surface of the gut.

The position of the air varies slightly from day to day, but even in a larva which had had no food for 6 weeks (being kept throughout under ordinary room conditions) there was no extensive movement. On the other hand, as soon as the larva is asphyxiated by flooding with water, it contracts its muscles violently, and within a minute all the finest tracheal endings become filled with air. On removing the water, fluid slowly rises again in the tracheal endings. Some of these results are illustrated in fig. 2.

The removal of the fluid from the tracheal endings after death, as described in the flea, does not take place in the mealworm. This could be explained by supposing that in the latter insect, post-mortem changes in the permeability of the walls of the tracheoles proceed more rapidly than the concentration of the body fluids by evaporation.

Tracheal Endings in some other Insects.

Observations similar to those described on the flea and mealworm have been made on a number of other insects. These can be noted very briefly.

In the cockroach (*Blattella germanica*) many of the tracheal endings are easily visible in the living insect. The terminal branches in all parts of the body contain fluid, though the air extends into finer branches in the case of the leg muscles. On asphyxiation (by flooding with water) the air rapidly extends into the finest branches, the movement of the air following closely upon the violent muscular contractions which asphyxiation causes. When the asphyxiation is relieved the fluid slowly rises to its original level, and this state of affairs persists until the death of the insect.

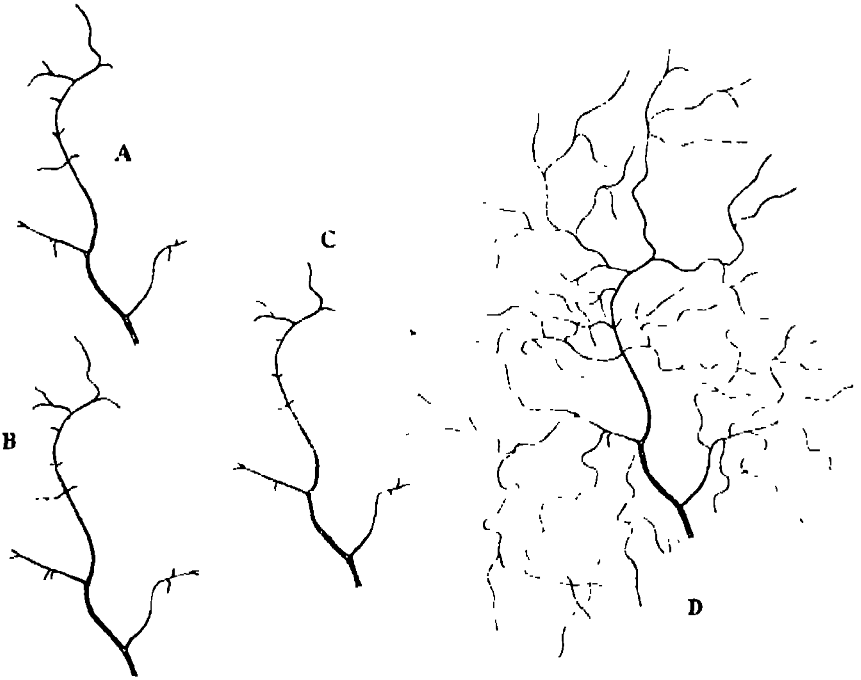

FIG. 2.—Tracheal ending on surface of gut of mealworm kept under coverslip without feeding. A, May 9, 1931; B, May 15, 1931; C, May 25, 1931; D, May 25, 1931, after asphyxiation by flooding with water.

In the bed-bug (*Cimex lectularius*) the finer tracheal branches over the surface of the gut contain fluid even when the insect has fasted for a fortnight or three weeks. When the bed-bug is asphyxiated it is extremely passive and makes no violent movements. It is doubtless on this account that no extension of the air in the tracheoles could be observed.

If a mosquito (*Aëdes* (*Stegomyia*) *argenteus*) is allowed to become gorged with blood, the tracheal endings in the abdomen can be readily seen by transmitted light against the distended intestine. The air extends only into relatively large tracheoles. Asphyxiation, by flooding with water, is followed by a downward extension of the air; but since the tracheæ are not visible in the fasting insect, it has not been possible to study the effect of desiccation.

Discussion.

The observations recorded in this paper prove that the changes previously described in the larva of the mosquito occur also in terrestrial insects. These observations raise several points of interest.

In the first place, since it has been shown (Wigglesworth, 1930) that it is osmotic pressure which determines the extent of the air in the tracheoles, the changes in position of the air will serve as an index of the osmotic pressure of the tissue fluids. And since there is no material change in the position of the air in the tracheoles, even after prolonged fasting, the osmotic pressure must be maintained at a more or less constant level.

This inference is borne out by the observations of Buxton (1930), who has shown that even after prolonged desiccation (keeping over sulphuric acid at 23° C. for a month) the proportion of water in the mealworm is unchanged. And light is thrown on part of the mechanism of the process in a recent paper by the present writer (Wigglesworth, 1931, *b*) in which it is held that the chief function of the rectal glands and rectal epithelium of insects is to recover water from the excreta and so maintain the normal composition of the blood. This function is particularly evident in the rectum of the mealworm.

These remarks apply to the tracheæ which lie on the surface of the abdominal organs. The tracheal branches to the muscles of the limbs, and, to a less extent, of the body wall, tend to contain air in their finest ramifications at all times; and the question arises why these branches are not subject to the osmotic pressure of the general tissue fluids?

The answer probably is that these branches, lying between the muscle fibres, are influenced more by the local changes in these muscles than by the osmotic pressure of the general body fluids. For the "open" circulatory system of insects cannot bring about a rapid mixing of the blood in all parts of the body. Consequently, the osmotic pressure between muscle fibres which are occasionally contracting will tend always to be somewhat higher than it is around the more exposed organs of the body. That there is no fundamental difference between the endings in muscles and in other tissues is shown by the observations on the tracheoles to the muscles of the body wall in the mealworm, which, during enforced rest, come to contain fluid.

This leads to the next point which requires further consideration: the possible physiological significance of the phenomenon. There can be no doubt that the replacement of water in the tracheoles by air will greatly improve the supply of oxygen to the tissues, since the diffusion of gas is so much more rapid in air than in water. The analogy of this process with the local control of the capillary circulation of vertebrates was pointed out in a previous paper (Wigglesworth, 1930). For in vertebrates the capillaries are mostly closed during rest, and are caused to dilate by the metabolites produced during activity. But this analogy is only superficial, because, whereas in vertebrates a restricted capillary circulation during rest is necessary to relieve the work of the heart, there is no apparent reason why all the tracheoles of insects should not be kept always filled with air.

The explanation of the phenomenon is to be sought probably in the physical nature of the materials composing the system rather than in the physiological needs of the organism. Thus, it is certain that the walls of the tracheæ are impermeable to water; and this is probably the case also with the larger tracheoles, for otherwise they would often come to contain fluid where they lie exposed in relatively dilute hæmolymph on the surface of the muscles, although the fluid in their finer branches within the muscle bundles had been removed by the higher osmotic pressure around their terminations. In other words, it is probably only the finest terminations whose walls are permeable to water.

Now where the tubes are larger they will need more resistant walls to save them from collapse when they contain air; and if it be supposed that this strengthening of the walls goes hand in hand with a loss of permeability to water, it can be readily understood why the permeable region of the tubes has so small a diameter that it is not kept filled with air by the normal osmotic pressure of the tissue fluids.

Summary.

It is shown that during rest the tracheal endings in a number of terrestrial insects contain fluid. During muscular contraction (*e.g.*, as the result of asphyxiation) this fluid is removed, presumably by the increased osmotic pressure.

The fluid is not removed during prolonged fasting under dry conditions (in the flea and mealworm). This suggests that the osmotic pressure of the blood of these insects is maintained during life at a more or less constant level.

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The Causative Organism in Infectious Ectromelia.

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With an Appendix by W. J. Elford on Filtration Experiments.

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[Plates 34, 35.]

I. Introduction.

Infectious ectromelia was described in 1930 by Miss J. Marchal.* The disease, which had not been recognised before, occurred in mice of the breeding stock at this Institute. Miss Marchal isolated various kinds of bacteria from the foot lesions of infected mice, but could obtain no experimental evidence that these organisms had causal significance. Ultimately she succeeded in passing the causative agent of the disease through bacteria-proof filter-candles and thereby proved it to belong to the group to which the term "virus" is applicable. The regularity with which ectromelia can be transmitted from mouse to mouse, and the ease with which the disease is identified, provided exceptionally good conditions for a closer study of the infective agent, and particularly for an attempt to discover whether it has the nature of an organism, which can be rendered visible by the microscopic and photographic methods, on the development of which I have been engaged for many years. It has the advantages that it produces typical "inclusion-bodies" in the cytoplasm of epithelial cells which it infects, and that, on the other hand, extracts containing abundant virus, as determined by the dilutions in which they convey the infection, can be obtained from infected organs, such as the liver, in which inclusion-bodies are not present, or are extremely rare. In the case of other virus infections forming inclusion-bodies, it has been proved, on the one hand, that an isolated inclusion-body can transmit the infection, and that its contents, when spread into a film and subjected to certain staining methods, contain minute particles on which the stain becomes preferentially deposited. Such results, however, do not provide very strong evidence as to the identity of such particles with the virus. In the case of the ectromelia virus I have been able to examine the intact, unstained, inclusion-bodies and their expressed contents, and to demonstrate the presence in closely-packed formation of minute refractile particles of uniform size and appearance, unstained and in the living condition. I have, further, been able to examine virus-containing extracts from tissues containing no inclusion-bodies, and to demonstrate the presence in these of minute particles indistinguishable from those with which the inclusion-bodies are closely packed. Of these particles, both from inclusion-bodies or in extracts from organs in which the virus is freely distributed, I have then been able to obtain photographic images with monochromatic ultra-violet radiation which enable the size and shape of the particles to be accurately described, and leave little room for doubt that they represent definite micro-organisms, of a size below the limits of ordinary microscopic resolution.

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* Marchal, J., 1930. 'J. Path. & Bact., vol. 33, p. 713 (1930).

II. Microscopical Methods.

I propose to leave for more detailed description, in a separate communication, the mechanical and optical apparatus which has been the subject of continuous modification and improvement in my laboratory for many years, and which has been devised for wider purposes than those of this investigation. It will be sufficient here to give a brief account of particular methods, so that the nature of the images observed and photographed may be clear.

The material examined being in all cases fresh, containing the virus in living condition, without fixation or staining, natural refractile properties of the objects observed are relied on for the production of images recognisable by direct vision or by photography. Two separate but related methods are used. In one the granular elements in a preparation are observed by means of a high-power dark-ground illuminator, the objective having the highest numerical aperture employable when the object is immersed in a watery medium. This limit is reached with an objective of 1·27 N.A., but in practice one of 1·22 N.A. has been found convenient. As a light source a quartz mercury vapour lamp provides a luminous area of uniform intensity and remains constant for long periods. Two microscopes are used, both are illuminated from the same source, and are provided with identical optical systems to ensure exactly comparable images. Thus infective material can be examined side by side with any desired control. Monochromatic green light of wave-length 5461 Å.U. is obtained by a suitable colour screen, and provides an illuminant whereby slight differences in intensity can easily be appreciated. The identification of bodies of a constant order of size or of luminosity is, therefore, comparatively easy, and with practice considerable accuracy is attainable. This method has been applied to membrane filtrates and concentrates, and latterly to the primary sand-filtrates, with the result that bodies can be identified by visual means although the image gives no real indication of size or form. This process is essentially preliminary ; it is the second method which is relied on for obtaining photographic images of sub-microscopic organisms by means of ultra-violet light. This also is a " dark-ground " method, but it can be used either for visual observation or for taking ultra-violet photomicrographs. The dark-ground illuminator is made of quartz in its refracting elements and of magnalium in its reflecting surfaces. It will, therefore, transmit visible or ultra-violet light, and, by a simple changing mechanism, either can be used. Suitable material having been selected, by the visual method first described, a preparation is made between quartz slide and cover glass, the image is focussed in visible light, and photographed in any suitable ultra-violet radiation, the necessary adjustment of focus being made in accordance with calculation.

A microscope of special design is used so that glass objectives can be used for all visual purposes and quartz objectives substituted, without interference with object or image, for taking ultra-violet photo-micrographs.

For ultra-violet photography the source of light has been an electric spark-discharge between cadmium electrodes. By means of quartz prisms and lenses, essentially an ultra-violet spectrograph, the desired line in the cadmium spectrum is separated and projected on to the posterior surface of the quartz-magnalium dark-ground illuminator referred to above. The wave-length commonly used has been $275\mu\mu$, but it is now possible to use shorter wave-lengths over a considerable range. In this way I have been able to obtain photographs with exposures so short as to reduce to a minimum the risk of change in the optical properties of the particles which are being photographed, through the action upon them of the ultra-violet rays.

Microscopical preparations are made from filtrates, concentrates or centrifuged deposits. For visual examination a small drop of the fluid to be examined is placed between two thin cover-glasses, the amount of fluid being such that none exudes from the edge of the cover-glass even with slight pressure. A thin layer is essential to avoid interference arising from superimposed images, and to reduce the mean free path of the granules. For ultra-violet photography the layer must be as thin as practicable, but this is obviously in large part dependent on the viscosity of the fluid. As quartz slides and cover-glasses, used for ultra-violet work, have optically worked surfaces, this is not so difficult as with glass, the fluid spreading more easily into a thin, even layer.

Immobility of the granules must be secured before photographs can be taken. In part the thin layer secures this, but after a short lapse of time, usually an hour or two, the granules attach themselves to slide or cover-glass. The best definition is secured when the granules are in optical contact with the under side of the cover-glass, but, provided that the thickness of layer is not more than two wave-lengths, either surface can be used.

III. METHODS OF PREPARING AND FILTERING SUSPENSIONS OF VIRUS.

Miss Marchal found that saline emulsions, made from the liver or spleen of a mouse dying of ectromelia, were very effective in transmitting the infection, when injected, in small doses of high dilutions, into other mice. We have accordingly used such emulsions for obtaining suspensions of free virus, which could be purified and concentrated by differential filtration.

Preparation of the Emulsion.—Only the preparation of the liver emulsion, used in most of our experiments, need be described. The mice, which were taken when just dead of the infection, or killed at an advanced stage of its progress, were all provided by Captain Douglas and Miss Marchal, by whom they were infected for our use with active virus.

0·6 gm. of the minced liver is placed in a sterile porcelain mortar and pounded to a smooth paste with a pestle. The paste is further ground with 2 gm. of washed and sterilized sand, of such grade that it will pass through a 60-mesh sieve. The mixture of liver pulp and sand is now thoroughly mixed with 40 c.c. of the fluid chosen for extraction, and the resulting emulsion transferred to a sand-and-pulp filter, which has meanwhile been prepared and tested.

Preparation and Use of the Sand-and-pulp Filter.—The correct and uniform preparation of such filters, for the preliminary removal of coarser particles, was found, as the investigation progressed, to be of increasing importance. Only when this stage of the preparation is properly carried out is material obtained in which the virus is sufficiently concentrated, and the particles which I regard as virus organisms sufficiently abundant, to enable these to be found readily by visual microscopic examination. A brief description of the exact routine of the preparation of these filters is accordingly desirable, though they do not differ in principle from those which others have used. (*Cf.* Gye and Andrewes.)

Sand sifted through a 30-mesh sieve is well washed and dried. The paper pulp supplied for filtration is broken into shreds, put into a dish, and covered with distilled water. After a few minutes the excess of water is poured off and the remainder expressed from the pulp as completely as possible. This process of soaking the pulp and expressing the wash-water is then repeated several times.

The filter is made in a glass tube, 7 inches in length and 1 inch in diameter, narrowed abruptly by a shoulder at its lower end. where it is joined to a narrow tube of 7 mm. diameter (see fig. 1).

To make the filter a pledget of absorbent cotton wool is first placed at the lower end of the wide tube. On this pledget is placed a layer of the washed pulp, on this a layer of the sifted sand, and on this again a second layer of pulp. Each layer of the damp pulp weighs 3 gm., and the sand layer is made of 7 gm. The three-layered filter is now compressed, by use of a plunger, formed of a rubber bung of suitable diameter attached to a glass rod. The compression should be such that, when the tube above the filter is filled with distilled water, the water passes through the filter, without added pressure, at the rate of 5 drops per minute.

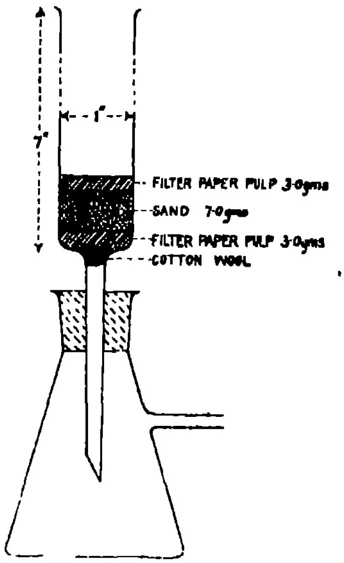


Fig. 1.

The finished filter is now attached to the flask in which the filtrate is to be collected, the upper end of the filter-tube and the beak of the flask are plugged with cotton wool, and the whole apparatus is sterilized in steam for 2 hours at 100° C. Autoclaving is not to be recommended, as it usually causes a change in the speed and efficiency of the filter.

The permeability of the filter is now again tested, by pouring into it 20 c.c. of the fluid which is to be used in making the liver emulsion—broth, Ringer's solution, or distilled water, as the case may be. It should pass, under its own weight, at about 5 drops per minute.

The emulsion of infected liver being placed in the filter tube, the apparatus is transferred to the cold chamber, at about 4° C., where filtration, without pressure, is allowed to proceed overnight. The filtrate is collected next day, and further prepared for examination by membrane-filtration, or centrifugation.

Filtration and Concentration by Membranes.—The differential filtration through graded collodion membranes was carried out by my colleague, Dr. W. J. Elford, who has elsewhere (1931) described his method of preparing membranes for this purpose, and gives, in an Appendix to this paper, some details of his application of the method to this investigation of the ectromelia virus. The object of the filtration through membranes has been to remove, from the sand-and-pulp filtrate, all particles above and below a certain narrow range of size, so chosen as to include the particles of the ectromelia virus. A membrane had to be found, therefore, of such pore size, that it would remove all coarser particles, including any bacteria which had passed the sand-and-pulp filter, and allow the virus to pass without serious loss. A second membrane had then to be found, of such pore size that it would prevent any virus from passing, but would allow particles of slightly smaller dimensions, and proteins and other substances in ordinary colloidal solution, to pass freely. By the use of these membranes in succession the virus-containing extract could first be purified and rendered bacteriologically sterile, and the purified virus could then be concentrated on the surface of the second membrane. It could be further purified, from particles of smaller size than those of the virus itself, by repeated washing on the second filter. Finally the virus could be washed off the surface of the membrane with a relatively small volume of clean water or saline solution, and thereby obtained in sufficient concentration, as determined by animal tests, to give a reasonable probability of observing the particles of the virus, in the extremely thin layers of fluid suitable for examination by the highest power of the microscope, always provided that the particles had a sufficiently different refractive index from that of the suspending medium, to be rendered visible by the dark-ground illuminator.

It was necessary to determine by trial the smallest average pore size of a membrane which would allow the virus to pass without loss, while removing all ordinary bacteria and larger particles, and the largest average pore size of a membrane which would retain the virus completely. Membranes of intermediate porosities would allow the virus to pass, with increasing loss as the pore size was diminished. By use of such intermediate membranes an approximate estimate could be obtained, as shown in Dr. Elford's Appendix, of the size of the virus particles. On the other hand, the difference in average pore size, between the two membranes used in clearing and concentrating the virus for microscopical investigation, could not be made very narrow, if sufficient virus was to be obtained to give a reasonable chance of recognising characteristic particles. It will be seen, from the detailed protocol given by Dr. Elford, that average pore sizes of 0·75 μ and 0·112 μ proved suitable for the first and second membranes respectively. The first twenty experiments of this kind were regarded as preliminary, only visual microscopical examinations being made of the different fluids, to ensure that the filtration was proceeding satisfactorily, and to obtain experience and confidence in identifying the particles characteristic of the virus by the visual method. Experiment 21, of which the detailed protocol is given by Dr. Elford, was the first complete one in which ultra-violet photo-micrographs were obtained of the particles so identified and located. Over eighty experiments, combining filtration and concentration with microscopical examination, have been carried out in all, control extracts from normal mouse livers being regularly treated in parallel and examined as controls.

Use of the Centrifuge.—Contrary to expectation, the virus particles, although of very small dimensions (about 0·13 μ to 0·14 μ in diameter) are thrown down by the centrifuge with comparative ease. If fluid containing the virus is enclosed in narrow tubes, centrifugation for 1 to 3 hours at 3,500 r.p.m. produces a deposit rich in the characteristic particles, and of high infective potency. The particles, though small, must be of relatively high density. Experience enables them to be identified in the deposit thus obtained from an extract subjected only to sand-and-pulp filtration, and their nature is confirmed by the ultra-violet photomicrographs.

IV. MICROSCOPICAL OBSERVATIONS.

These have been applied concurrently to the separated cytoplasmic inclusions from the infected epidermis, and to the virus-containing extracts prepared and concentrated as above described.

1. *Observations on Cytoplasmic Inclusions.*—These bodies, as seen in stained preparations of epithelial tissues (skin, tongue, pancreas) from mice infected with ectromelia, appear to be structureless masses of variable size. A tendency to granularity of their contents can be seen in a very small number of the inclusions in such preparations, but there is no regularity of structure. In frozen sections cut from fresh material the inclusions present quite a different picture. My own observations have been made entirely on such fresh material.

Mice were inoculated with the virus on the soles of the feet. When the disease had progressed to a suitable stage (usually after 4–5 days) the mouse was killed, and the hairless pad of the infected foot removed with scissors. Frozen sections of the fresh pad were cut vertically to the surface, and kept in Ringer's solution. If such sections are transferred to distilled water in a narrow tube, and kept in an incubator at 37° C. for a few hours, or overnight at 0° to 4° C., inclusion-bodies become detached from the macerating epidermal cells and settle to the bottom of the tube. Slow maceration in the cold room is preferable to shorter treatment in the incubator, since bacteria multiply rapidly at the higher temperature.

If a fresh section is directly examined, without preliminary maceration, the inclusion-bodies present a striking appearance under a low power of the microscope, with a suitable dark-ground illuminator, being easily recognised in their natural position in the section. With a suitable intensity of the light source, an apochromatic objective of 16 mm. equivalent focal length, and a low-power dark-ground illuminator, the picture is striking and characteristic. The material of the section, apart from the inclusion-bodies appears white; the intensity of the illuminant should be just sufficient to give it this degree of visibility. The inclusion-bodies now appear to have a definite, sky-blue colour, and stand out in strong contrast to the white background of the other structures. By this appearance the inclusions can be identified easily and with certainty; even a single inclusion in a thin section, or one which has become detached, can easily be recognised. (Figs. A and B, Plate 34.)

The blue appearance of the inclusions when thus illuminated is not due to a true colouration of their substance. It is an optical effect, due to the regular distribution in an optically homogeneous matrix of small particles with a different refractive index. Whenever a beam of light passes through a transparent homogeneous medium, in which particles of constant size, and of constant refractive index, differing substantially from that of the medium, are regularly disposed, the light will be scattered by the particles. If the particles are small in relation to the mean wave-length of the beam, the scattered light will be predominantly blue, and its greatest intensity will be at right angles to the beam. The phenomenon is similar to that known as the "Tyndall effect," and its theoretical basis has been formulated by Rayleigh (1871). It is neither possible nor desirable to illuminate a section by a beam at right angles to the axis of the microscope; but a low-power dark-ground illuminator provides a sufficiently close approximation to this optical condition.

In practice this optical method of differentiating inclusion-bodies from the rest of a fresh section has proved of value. Not only mature inclusions, but small, presumably early ones, are recognised with the same ease. It should be noted that, in using this method of observation, it is important that the material should not be macerated in distilled water, or otherwise treated so as to alter the disposition of the granules in the homogeneous matrix. In sections preserved for some weeks in 0·5 per cent. formaldehyde the blue colour of the inclusions can still be seen.

The ectromelia inclusion-bodies, when fully developed, are oval bodies about 12–13 μ in length and 6–7 μ in breadth, and their size and shape vary but little when they are mature. Fig. B shows their appearance in the fresh section. There is no evidence of a limiting membrane of any kind; the bodies readily coalesce and form large masses if they are macerated in distilled water for a few hours. This tendency probably explains the variation in the size of the structures described as inclusion-bodies in fixed and stained sections, some of which probably represent masses formed by coalescence of the original bodies, at some stage of the process of fixation. The natural bodies, as seen in fresh sections, appear to behave like colonies of organisms, growing rapidly for a short time, until a certain maximum dimension is reached. The organisms, assuming the regularly distributed refractile particles to be such, are in this instance enveloped in an optically clear matrix, which appears to increase with multiplication of the organisms, and may be an essential condition of it in cells of certain types. At the earliest stage of its formation, at which an inclusion-body has been recognised as such, it is spherical in shape (fig. C). As it enlarges and approaches maturity it becomes somewhat elongated.

Structure with Visible Light.—With suitable dark-ground illumination and an objective of the highest aperture, it is possible to see and to photograph the internal structure of an inclusion-body in the early stage of its development, when it is small in size and spherical in shape. In such a body, photographed under these conditions, it is easy to see that the substance contains refractile granules of uniform size in large numbers, but not so thickly set as to prevent their being perfectly resolved by the microscope. As the inclusion-bodies approach maturity, and the granules become more closely packed, the greater opacity prevents satisfactory resolution without preliminary treatment. To display the granules in these more mature inclusions, the latter are macerated in distilled water for a short time, to soften the matrix, and then gently pressed between two cover-glasses.

Fig. D shows the appearance which can be seen and photographed after such treatment. It will be seen that the granules have been spread out, without altering their positions in relation to one another in the matrix. The regularity of their disposition, producing a resemblance to what bacteriologists call an "impression preparation" will be clear; the uniformity of arrangement strongly suggests that which occurs in a colony of bacteria.

Some difficulty was encountered in obtaining the granules completely separated from the homogeneous matrix. This was ultimately accomplished, however, by variation of the time and temperature of the maceration process. The result of such a successful separation is shown in fig. E, in which the uniform size of the granules is obvious, where they have been separated and spread into an even film.

Even with the use of visible light, the similarity in size and refractility of these granules from the inclusion-bodies to those which has been observed in filtrates from infective organ extracts, and regarded as probably representing the virus, had become clear. Figs. E and F enable a comparison to be made of the particles from these two sources, photographed under identical conditions. No microscopical method, however, using light in the visible spectrum, is capable of showing the exact form and size of particles such as these. Their size lies below the limits of accurate resolution with light of such long wave-length; the images obtained are circular discs, whatever the shape of the particles, and their apparent size is dependent on the intensity of the illumination. Photo-micrographs of true images of the granules contained in the inclusion-bodies have been obtained with ultra-violet rays of suitable wave-length. (See figs. R and S.) These will most conveniently be described, however, in comparison with the similar photographs of the particles observed in virus-containing extracts.

2. *Observations on Virus-containing Liver Extracts.*

Observations with Visible Light.—The methods of freeing the extracts from particles of larger and smaller sizes than the range within which those of the virus are found, and of concentrating the latter, have been described in an earlier section. A further optical differentiation can be effected, in the use of visual microscopy, with the dark-ground illuminator, by adjusting the intensity of the illumination in the manner described. By such means it has been found possible, in a purified and concentrated virus-containing filtrate, to recognise, with some confidence, particles of a characteristic size and luminosity. The images so obtained give, indeed, no real indication of size, and none at all of form. Their recognition, however, is an essential preliminary to critical examination by ultra-violet photography, since the objects to be photographed must be located and brought into focus in a known visible wave-length, before they can be focussed by calculation for the ultra-violet wave-length with which the photograph is to be taken. As a result of experience, I can state that practice enables one to discriminate between virus particles and other particles in the virus-containing filtrate in the preliminary visual observation, so that the subsequent ultra-violet photograph seldom shows the selected object to have other than the characteristic size and shape. As stated above, it has become possible, with experience, thus to select for ultra-violet photography particles, or groups of such, in the deposit obtained by centrifugation from a filtrate which has been passed only through sand and pulp, and has not been further subjected to differential filtration through membranes.

Ultra-violet Photography.—The ultimate aim of the whole investigation has been to obtain, and to record by photography, critical images of particles which could with confidence be regarded as representing units of the infective virus, using a wave-length in the ultra-violet sufficiently small in relation to the size of the particles. From such images the true size and shape of the particles could be determined, and an idea could be formed as to the likelihood of their having the nature of true micro-organisms. This method of examination has been applied to the granules contained in inclusion-bodies, both *in situ* and after expression from the macerated body and liberation from the matrix. It has also been applied to the particles, identical in appearance with the inclusion-granules when examined with the dark-ground illuminator and visible light, occurring in the suspensions obtained by differential filtration or centrifugation from virulent liver extracts.

Elford calculates, from the filtration results described in the Appendix, that the units of ectromelia virus have a diameter not greater than 0·15 μ, or less than 0·1 μ. For objects of this size, the use of rays of wave-length 0·275 μ for illumination should enable a true image to be obtained. There is a sufficient margin of efficiency in this case to ensure a close resemblance between object and image. Good, sharp photographic images have, in fact, been obtained, and measurements of them indicate a size within the range indicated by Elford, being, in fact, 0·13–0·14 μ. The photographic images of the particles in the inclusion-bodies, and of those selected from the virulent liver extract, are completely identical in appearance, as may be seen in figs. K, M, N, O, L, R, S, Plate 35. A common description may, accordingly, be given for the particles from both sources. They have definitely the appearance of minute micro-organisms, and I propose to describe their morphology as that of the organism of ectromelia.

Morphology of the Causative Organism of Infectious Ectromelia.

Although a complete account of the morphology of such a minute organism cannot be given at this stage, there are certain characteristics observable in the numerous ultra-violet photographs which, together with some which can be seen directly with visible light or observed by other means, justify a general description. In form the organism is coccoid. Pairs are common, and elongation is evident as a preliminary to division. Isolated organisms are spherical and highly refractile, and the refractivity appears to increase with shortening of the wave-length employed for illumination. The small mass is apparently associated with high density, as indicated by the ease with which they are thrown down by centrifugation in narrow tubes, even at 3000 r.p.m. Reproduction is by binary fission, but the period of elongation, before division occurs, is short, the final separation taking place quickly and sometimes suddenly. The separating elements often appear to have a connecting filament, which on rare occasions has actually been seen. If one organism of a pair becomes attached to slide or cover-glass, the other is often seen to move in a path which suggests a connection between the two.

The ultra-violet photographs suggest that there is a cell wall, indicated by a more highly refractive outline. There is, however, an obvious possibility that, in cells so minute as these, the surface is so large in relation to the small mass that the cell wall is not a separate structure, but only such a limiting membrane as would be produced by effects of surface tension. Interfacial phenomena are likely to be more effective in determining the morphology of such minute organisms than in the case of larger ones. A study of the original negatives indicates that, as the cell elongates before division, there is an increased density of the more refractive cell membrane at the poles, and a thinning, suggesting a condition of strain, in the middle. In larger organisms bipolar bodies are common, and are regarded as due to local change in the quality or density of the cytoplasm. In the case of organisms so minute as these, however, it seems possible that the appearance of bipolar density is, again, only an effect of interfacial phenomena.

DISCUSSION.

The evidence here presented for the identification of the minute bodies demonstrated by the microscope, as actual units of the infective agent of ectromelia, appears to be as complete as any which can be obtained when dealing with a virus, unless it should prove possible to cultivate it in an artificial

Fig. D.—Infectious Ectromelia. × 1250. Mature inclusion-body in distilled water showing regular distribution of organisms in matrix.

Fig. E.—Infectious Ectromelia. × 1250. Free organisms from inclusion-body after maceration in distilled water.

Fig. F.—Infectious Ectromelia. × 1250. Same material as fig. M, Plate 35. The large white objects are sand grains.

Ultra-violet Light Photo-micrographs. (Figs. G and H. *Chr. prodigiosum.* × 3200.)

The photo-micrographs of *Chr. prodigiosum* are included to provide standards of reference for estimation of relative sizes, and to suggest the type of image obtained of these organisms when photographed in the living state with ultra-violet light. Two photographs are included to show the effect on the image when the objective is focussed on different planes. In one (fig. G), the cell wall is in focus, in the other (fig. H), the cell contents are better defined. A similar difference is suggested between fig. M and fig. N.

Plate 35.

Ultra-violet Photo-micrographs.

Fig. K.—Infectious Ectromelia. × 3200. Preparation made from a membrane filtrate of infective liver emulsion (see Exp. 21).

Fig. L.—Infectious Ectromelia. × 3200. Preparation made from a membrane filtrate of infective liver emulsion concentrated by further filtration through a less permeable membrane.

Fig. M.—Infectious Ectromelia. × 3200. Centrifuged deposit from sand-and-pulp filtrate of infective mouse liver. Preparation made from same material as fig. F, Plate 34.

Fig. N.—Infectious Ectromelia. × 3200. Preparation as fig. M, but showing the colony-like grouping that frequently occurs.

Fig. O.—Infectious Ectromelia. × 3200. Preparation as fig. M, but from infective spleen.

Fig. P.—Infectious Ectromelia Cytoplasmic inclusion-body. × 2000. From frozen section of mouse foot. Individual organisms are seen on outer edge.

Fig. R.—Infectious Ectromelia organisms separated by maceration from inclusion-body matrix. × 3200.

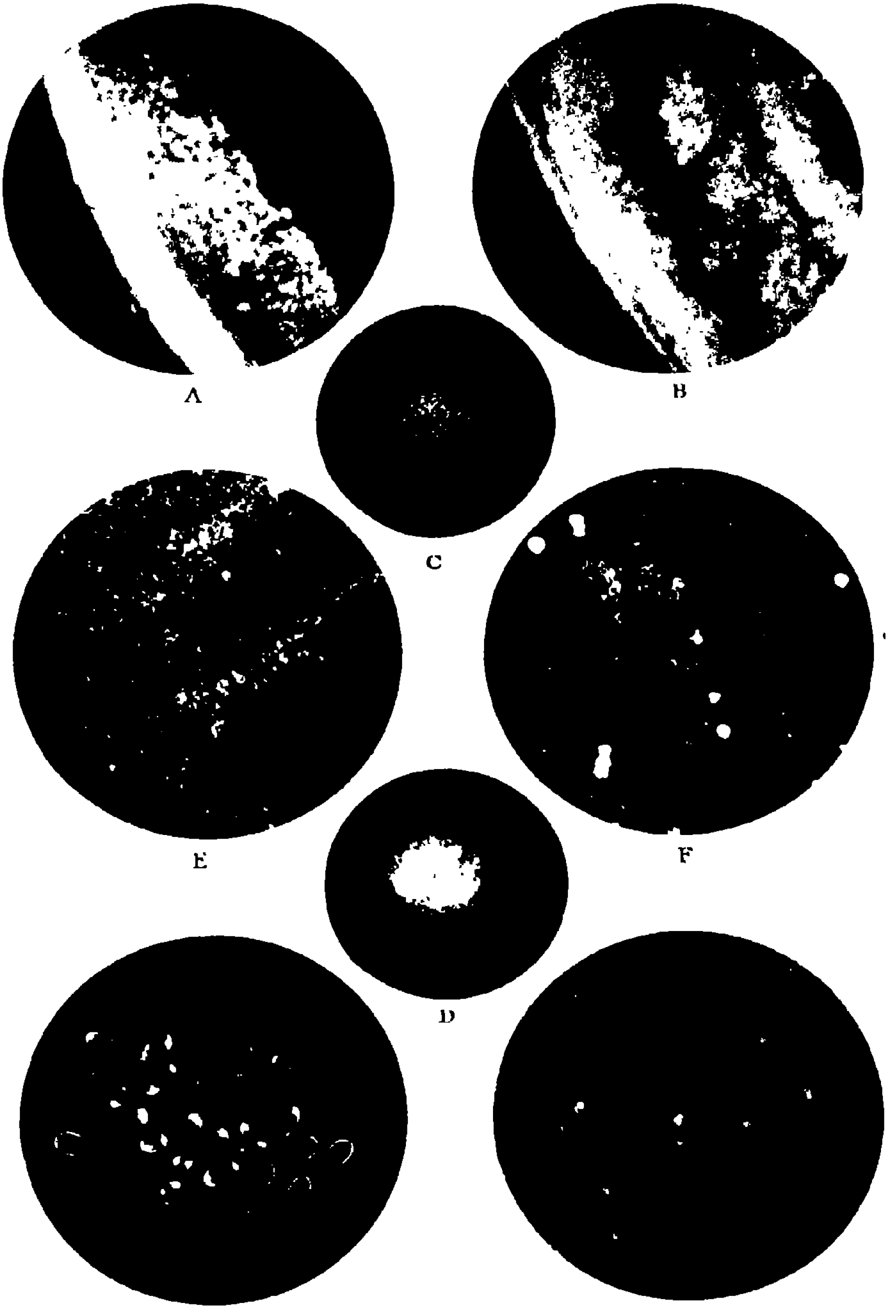
Fig. S.—Infectious Ectromelia. As fig. R. × 3200.

Figs. R and S are preparations from the same material as shown in fig. E, Plate 34.

APPENDIX.—*Filtration Experiments with Virus of Infectious Ectromelia.*

By W. J. Elford.

The primary demand upon any filtration method, as an adjunct to microscopical studies, is that it should provide a means of fractionating from a polydisperse system particles which within certain limits are of a definite order of size. To accomplish this it is essential to have a series of graded filters of



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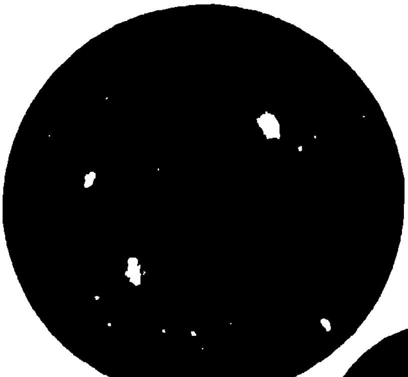

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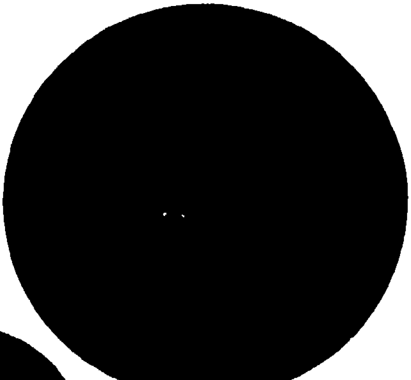

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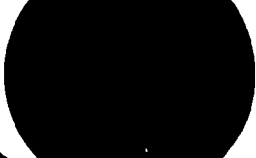

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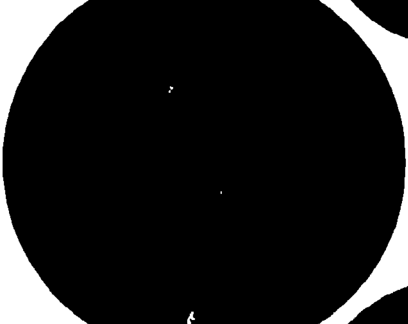

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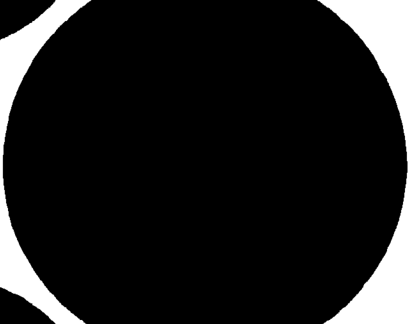

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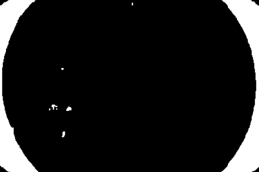

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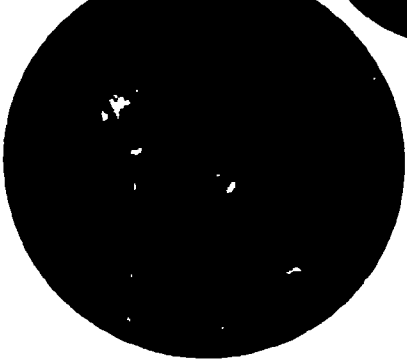

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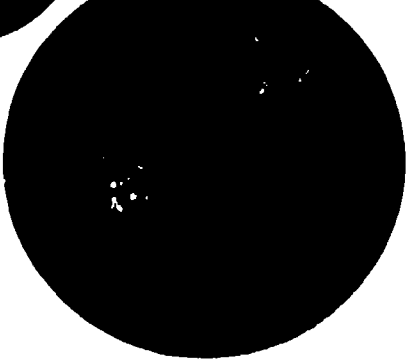

S

known porosities, capable of being reproduced with constancy. Then, by adopting a carefully regulated filtration technique, graded filtrates can be obtained, the particle size of dispersed material in any one of which is determined by the grade of filter employed. The use of such a method in conjunction with microscopical examination of filtrates provides at once a direct method for the analysis of disperse systems. This, of course, assumes that the disperse phase is resolvable by the optical methods available.

Mr. Barnard has already indicated briefly the part played by filtration in the present study on the virus of infectious ectromelia. I propose to describe in more detail the lines along which the filtration experiments have been conducted, and particularly the steps in the development of the general experimental method.

Membranes.

A new series of graded collodion membranes has been used, the preparation and properties of which have been described in a recent paper (Elford, 1931).* Reference should be made to this paper for particulars. It suffices to state here that uniform reproducible membranes, graded in porosities from 2–3 μ downwards to 10 μμ or less in estimated pore diameter, are available.

Filtration Apparatus.

The membrane, supported on a perforated plate, is assembled in an ultra-filter (see fig. 2). The design of the latter differs slightly from that described in an earlier work (Elford, 1929).† An hexagonal nut and screw thread replaces the three nuts and bolts as the means of clamping the component parts of the filter together. This automatically ensures an evenly distributed pressure upon the rubber washer, making an effective joint with the membrane; and, further, it greatly facilitates the final operation in assembling the filter. An important feature, not to be overlooked, is the slot-locking device (× in fig. 2), which prevents any shearing stress being transmitted to the membrane in the process of clamping. Any risk of tearing the membrane is thereby eliminated.

The component parts of the ultra-filter are sterilised in the hot air oven, while the membranes are subjected to "steaming." The complete filter is assembled in the sterile atmosphere of an ultra-violet light chamber (Elford, *loc. cit.*).‡

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* 'J. Path. & Bact.,' vol. 34, p. 505 (1931).

† 'Brit. J. Exp. Path.,' vol. 10, p. 126 (1929).

‡ 'J. Path. & Bact.,' vol. 33, p. 713 (1930).

Experimental.

The first step in the filtration study was to determine the filtration end-point for the virus, *i.e.*, the limiting grade of membrane which permits the virus to pass.

The starting material was a sand-and-pulp filtrate of a 1 per cent. emulsion in broth of infected mouse liver. A method for standardising the sand-and-pulp filters, which Mr. Barnard has described, was introduced to ensure that the initial material should be comparable in all experiments. This filtrate, when passed through a membrane of A.P.S. (average pore size) 0·75 μ at 20 cm. mercury pressure, gave an actively potent ultra-filtrate, which invariably titrated to 1 in 100,000 when tested by inoculating 0·2 c.c. into the foot of a normal healthy mouse. This membrane effectively retains *B. prodigiosus*, and all filtrates were found to be sterile by cultural tests. Adequate confirmation is thus afforded of the work of Marchal (1930), who, on the evidence of its passage through bacteria-tight filter candles, placed the causative agent of this disease in the filterable virus group. The virus continued to pass

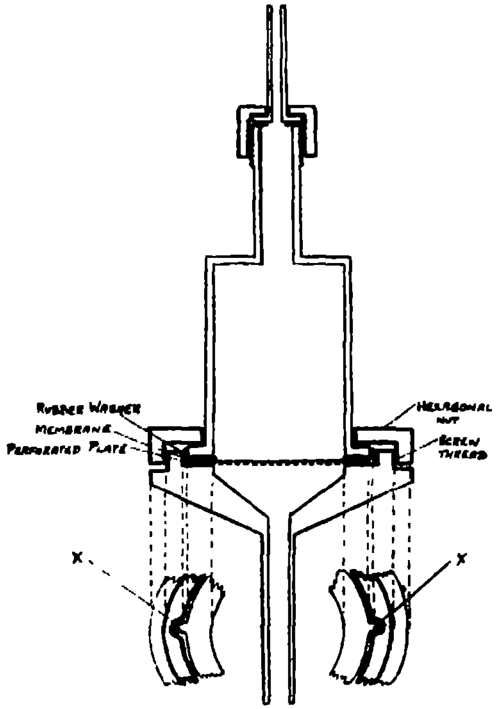

FIG. 2.

readily all membranes down to A.P.S. 0·3 μ, when a range of pore size was reached where filterability is affected greatly by the conditions of experiment, particularly the filtration pressure. The definite end-point of filtration of the virus was found to be 0·2 μ, a membrane of this grade effectively retaining the virus under all conditions tried. (Table I summarises results.) This corresponds with the end-point of filtration of the organism of bovine pleuro-pneumonia. On the basis of numerous experiments with this latter organism in particular, and with standardised metal sols, I have been able to deduce a relation between the size of the pores of a membrane, and the size of the particles which it allows to pass. I deal with this relation more fully in a forthcoming paper. Here it may be stated that, on this basis, I calculate that the infective particles of the ectromelia virus are not greater than 0·15 μ or less than 0·1 μ in diameter. It follows, therefore, that, with an optical system capable of resolving structures of the order 0·1 μ, the virus of infectious ectromelia, if existing as elementary particulate units, should be discernible

Table I.

No. of Experiments.	Membrane Grade A.P.S.	Filtration Pressure.	Virus Activity in Filtrate.	Remarks.
10	0·75 μ	30 cm. Water 20 cm. Mercury 76 cm. ,,	+ + + + + + + + +	*Filtration Conditions*— p_H — 7·5–7·6. Volume of filtrate 10 c.c., 1 sq. cm. membrane surface.
3	0·65 μ	76 cm. Mercury	+ + +	
8	0·60 μ	20 cm. Mercury 76 cm. ,,	+ + + + + +	
10	0·50 μ	20 cm. Mercury 76 cm. ,,	+ + + + + +	*Virus Activity*— + + + Mouse succumbs to disease showing typical symptoms within 7 days. + + Mouse succumbs in 7–10 days. + Mouse succumbs in 10–14 days. ∓ Result sometimes —, sometimes +, more frequently —. ± Ditto, more frequently +
14	0·40 μ	20 cm. Mercury 76 cm. ,,	+ + + + +	
7	0·3 μ	20 cm. Mercury 76 cm. ,,	+ + +	
10	0·25 μ	30 cm. Water 20 cm. Mercury 76 cm. ,,	∓ ±	
6	0·2 μ	20 cm. Mercury 76 cm. ,,	—	
20	0·15 μ	20 cm. Mercury 76 cm. ,, 3 atmos.	— — —	

in infective filtrates. It will be seen that Mr. Barnard's direct measurement of the particles photographed by him, and indentified by him as the virus organisms, falls in this narrow range.

Concentration of Virus.

From the standpoint of microscopical study it is eminently desirable to have the virus present in high concentration, to facilitate its detection. The evidence of the present study indicates that, to detect the virus with reasonable certainty, it must be present in such concentration as to remain infective in a dose of 1 c.c. after being diluted 10^4 times ; *i.e.*, it must contain at least 10^4 minimum infective doses per c.c. Filtration has been able to assist here. The procedure adopted is as follows :—

The sand-and-pulp filtrate is filtered through a permeable membrane of A.P.S. 0·6–0·75 μ, to furnish a virus-active filtrate free from ordinary bacteria. This filtrate is then concentrated by using a membrane known to effectively retain the virus (A.P.S. 0·1–0·2 μ). Noting the volume of the charge placed in the filter, nine-tenths of this is collected as filtrate. The residue will contain all the virus originally present. A tenfold concentration may be effected in this way. The following protocol of an experiment illustrates this.

Protocol. Experiment 21.

The mouse was killed in early stages of the disease, the liver was consequently less pale and spotted than in a typical advanced case.

Material—

1 per cent. broth emulsion of infected liver, filtered through sand-and-pulp filter to remove coarse tissue debris.

Permeable Membrane Filtration—

Membrane Grade	A.P.S. 0·75 μ.
Filtration Pressure	20 cm. mercury.

Ultra-filtrate referred to as Stock Filtrate.

Concentration of Stock Filtrate—

Membrane Grade = A.P.S. 112 μμ.
Filtration Pressure = 3 atmos.
Filter Charge = 10 c.c.
Filtrate Volume = 9 c.c.
Residue ,, = 1 c.c.

Control Animal Tests—

In each test 0·2 c.c. was inoculated into the foot of a healthy mouse.

Undiluted stock filtrate ..	Mouse showed typical foot symptoms on 6th day.
1/100,000 dilution stock filtrate	Negative. Mouse normal.
Residue undiluted	Mouse showed positive foot reaction on 3rd day.
1/100,000 dilution residue ..	Mouse showed positive foot reaction on 16th day.
Ultra-filtrate (112 μμ membrane) undiluted ..	Negative. Mouse normal.

Conclusion.—The residue is definitely more potent than initial stock filtrate and control animal test would suggest probable tenfold increase.

Note.—The fact that the stock filtrate gave no reaction in 1/100,000 dilution is most likely due to there being less virus in the original tissue at an early stage in disease.

Purification of Virus by Washing.

Virus obtained by extracting infected tissue with a suitable liquid medium, is always associated with quantities of tissue proteins. The presence of the latter is very undesirable for microscopical studies, and, indeed, for other studies also, particularly in immunity experiments. Endeavour was therefore made to prepare a suspension of virus which was free from a large percentage of these proteins. The procedure followed was exactly that in concentrating the virus. After filtering nine-tenths of the volume, the residual one-tenth was again diluted with the wash liquid—water, physiological saline, or broth, as desired. Nine-tenths were again filtered and the process repeated until the filtrate no longer gave any protein reaction. The residue, either in concentrated form, or made up to the original volume, contained virus with only slight traces of additional protein. The improved optical transparency of the medium is a great help in microscopical study, resulting in a sharper image being formed.

The activity of the washed virus was comparable with that of the original, but in titration experiments a drop in titre of approximately one-tenth was usually found. This is most probably due to the aggregation of the virus into clusters at the membrane surface, during the washing treatment.

This method of obtaining virus free from protein, although somewhat laborious, should also prove useful for the study of immunity reactions.

The quantitative aspect of the filtration study of the virus of infectious ectromelia, with the results of titrations of the virus at various stages by means of animal tests, must be deferred to a later paper. I should like to acknowledge here, however, my indebtedness to Captain S. R. Douglas and members of his department, who have throughout the course of this investigation supervised and conducted the control animal experiments.

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Micro-Operations on Cells in Tissue Cultures.

By Robert Chambers and Honor B. Fell (Beit Memorial Research Fellow).

(Communicated by Sir William Hardy, F.R.S.—Received August 17, 1931).

(From the Strangeways Research Laboratory, Cambridge.)

[Plates 36, 37.]

Introduction.

The application of the micromanipulative technique to the study of cells in tissue culture has for a long time offered an interesting, though difficult, field of research. Thus far, comparatively little has been done, the most notable contributions being those of Levi and of Péterfi and co-workers (Levi, 1926; Péterfi and Olivo, 1925; Péterfi, 1927; Péterfi and Kapel, 1928). Péterfi has also written a special paper on the technique (1927). The main deterrent in this work has been the lack of sufficient ease in the accurate and continuous control of the microneedles under the conditions required.

Recently it has been possible to increase the steadiness of the Leitz instrument by a system of flexible shafts to operate the adjustment screws of the micromanipulator, so that no vibrations of the hand are imparted to the rigid parts of the apparatus to which the needles are attached. Another feature which has greatly enhanced micromanipulative work under high powers is the use of dark-ground illumination made possible by a special condenser recently manufactured by E. Leitz. The upper surface of the condenser is broad, and a large, shallow cone of light emerges from its rim. A small sector of the rim has been cut away and the central region of the top of the condenser hollowed out to permit the introduction of microneedles beneath the cone. The tissue is operated upon in a hanging drop suspended in a moist chamber which encloses the condenser. Remarkably good resolution was obtained with this condenser, the illumination being such that the finer cytoplasmic granules, mitochondria and the delicate cell-boundaries were plainly visible.

Technique and Material.

The source of light was the Pointolite. Ordinary transmitted light was used occasionally, but most of the work was done with the dark ground.

The principal objectives used were the 10a and 12a oil immersion of Leitz.

Cultures of the following tissues were made : —Choroid, sclerotic and retinal pigment epithelium, mesonephros, intestine, skeletal (limb) muscle and spleen from 9-day fowl embryos and periosteum from 14-day embryos. The tissues were grown by the ordinary hanging drop method in a fluid medium which was prepared as follows :—Two parts of embryo extract were mixed with one part of plasma and the mixture was allowed to clot. The clot was then cut up into fragments and incubated for 30 minutes at 37·5° C. A considerable quantity of fluid exuded during incubation and this was removed with a pipette and used as the culture medium. This fluid medium is always employed at the Strangeways Laboratory for studying cultures under dark-ground illumination where the presence of fibrin would confuse the light. Every 48 hours the cultures were washed with embryo extract and fresh medium was added. The cultures were generally used 24 hours after the second change of medium. In order to facilitate adhesion of the growing tissue to the coverslip the explant was mounted in a rather shallow drop of medium. When the coverslip was removed and placed on the moist chamber of the manipulator, more fluid usually was added to minimise the effect of evaporation.

The moist chamber was of a special form adapted for use with the dark ground condenser. All the experiments were made with the microscope and manipulator in a thermo-regulated warm box at 38—39° C.

On the stage of the manipulator in the warm box the tissue did not remain in a normal condition for much longer than half an hour. The experimental work, therefore, was limited to the first fifteen or twenty minutes and the preparations were always discarded when they showed the least sign of deterioration within that time. This precaution necessitated the use of cultures in large numbers and we take this opportunity of expressing our appreciation of the valuable and skilful assistance of Mr. V. C. Norfield, the technician of the Laboratory, who kept us supplied with an abundance of splendidly growing material in a liquid medium.

All the observations recorded in this paper were made simultaneously by two observers through a double demonstration eyepiece and no conclusions were reached unless agreed upon by both.

This paper deals with the microdissection, mainly puncturing experiments, of a variety of cells in tissue cultures.

In the microdissecting chamber the first signs of deterioration of a culture were a slowing down and stopping of cellular and intracellular movement, followed by a faintly perceptible increase in the opacity of the cells. This was best seen under dark-ground illumination. As a matter of fact, the dark-ground studies proved to be most valuable not only because of the splendid detail of structure revealed, but also because of the ease with which it enabled one to detect the appearance of fine coagulation granules in the cytoplasm when deterioration was on the way. Frequently, in otherwise healthy-looking cultures with cells growing out in sheets, the peripheral attachments of the sheets would give way in places resulting in a general breaking loose of all the peripheral attachments and the retraction of the entire sheet of cells into the main body of the explant. This phenomenon usually occurred when visible signs of deterioration in the cells had set in. We may, therefore, consider the loss of adhesiveness as one of the usual signs of impending death. Sometimes, however, the detachment was due plainly to mechanical causes of undue stress, set up, for example, by tilting the drop or by loosening certain attached areas with a needle. Indeed, the principal difficulty of operating on one of the outlying cells at the periphery of the sheet lay in the fact that the cell operated upon, by losing its adhesiveness and becoming detached, tended to place too great a strain on the neighbouring cells with the result that the sheet would give way and retract, frequently carrying with it the cell being operated upon.

The various types of cells studied were fibroblasts, resting and mitotic, macrophages, pigmented epithelial cells of the retina, intestinal epithelial cells and skeletal myoblasts.

Experimental.

1. *Fibroblasts.*—A well spread out fibroblast with the sweeping lines of its borders and a cytoplasm containing shimmering fat-like globules and

sinuous mitochondria in slow, constant motion gives one the impression of a very delicate fluid body.

With manipulation, however, the effect is one of surprising stiffness. For example, a cell spread out on the coverslip was pulled loose on the tip of a needle. The pseudopodia remained partly extended as the cell was moved to and fro in the liquid medium. A minute later the needle with the cell attached to it was lowered out of the hanging drop and immediately returned, whereupon the material of the cell was found to be glued as a rounded mass to the tip of the needle.

The stiffness of the fibroblast is mainly due to its enveloping membrane particularly along the sweeping, curved borders typical for an extended fibroblast. Fine strands of material can be drawn out of the border on the tip of a needle without disturbing the general contour. The strands when released recede and there is no sign of resulting injury. If, however, the membrane is pricked and torn quickly the uniformity of the outline disappears in a wave spreading from the site of the tear. The exposed cytoplasm may persist as a coagulum, the boundary of which is quite different from the original boundary of the living cell.

An elongated pseudopodium with a spread-out tip was carefully pinched across its middle with the cylindrical side of a horizontal needle (text-fig. 1). As the pinching was increased the internal granules receded in both directions and the pinched region gradually narrowed to an attenuated strand. With the release of pressure the base of the strand swelled in waves which moved along the strand and progressively thickened it until after several minutes it had attained and surpassed its original size.

The reaction of the fibroblast to mechanical irritation is always very slow. The following are typical examples. An expanded, triradiate cell was viciously probed with a needle. A minute passed by before one of the pseudopodia was slowly withdrawn. After several minutes, a new pseudopodium grew out in the same direction as the first. In another case, the side of an extended pseudopodium was considerably indented by poking with a needle. This caused a few granules at some distance from the indented region to move. After a short time, the pseudopodium became detached and retracted, at first slowly and then with increasing rapidity, leaving cytoplasmic remnants stuck to the glass. After several minutes the cell was found to be spread out again with large new pseudopodia and with good intracellular movement.

The centrosphere region frequently was plainly visible, lying against a slight indentation on one side of the nucleus. This area could be punctured repeatedly with no visible effect either on it or on the cell as a whole. Pushing it against the nucleus tended to compress the centrosphere area without

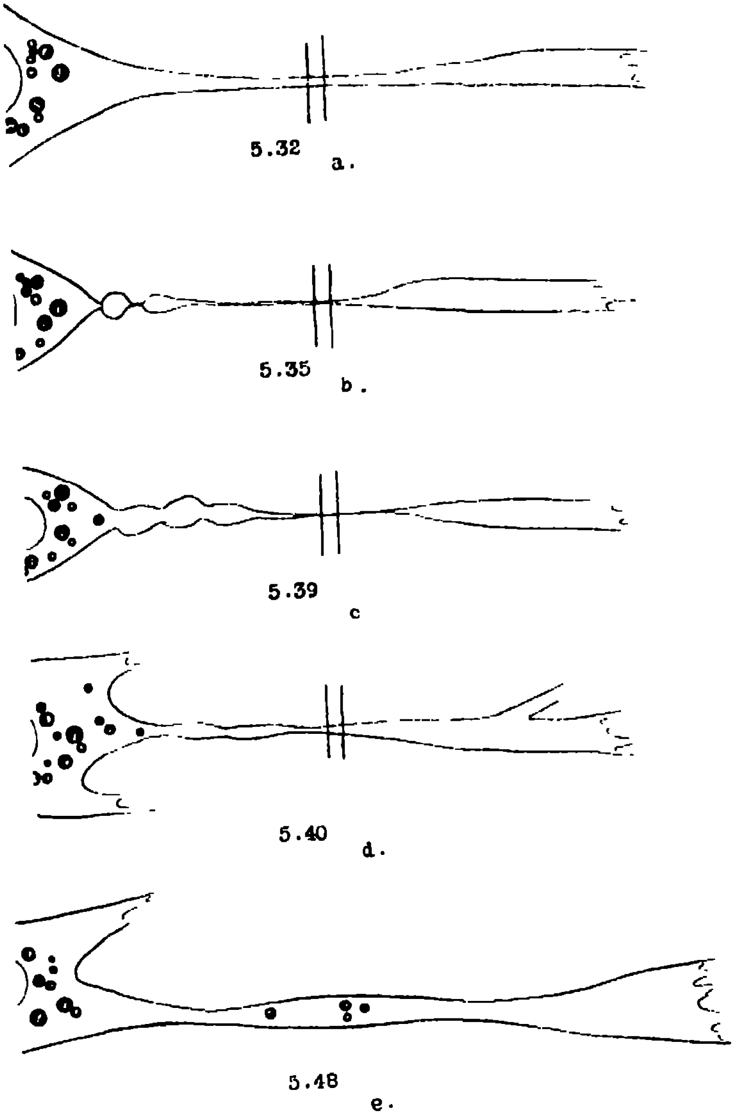

Fig. 1.—The effect of pressure on the pseudopodium of a fibroblast. (*a*) Immediately before pressure. (*b*) During the application of pressure. Note the attenuation of the strand. (*c*) Immediately after release of pressure. The thread of cytoplasm is adherent to the needle. Note the wave-like swelling at the base of the pseudopodium. (*d*) The wave-like swellings have passed along the strand which has become thicker. (*e*) The strand has now enlarged to its original size.

producing any such effect on the nucleus. On the other hand, the following observation indicates that the centrosphere is a definitely localised region of an appreciable stiffness. The nucleus of an expanded cell was injured by puncturing. The resulting cytolysis caused a hole to be formed through the flattened cell. The hole quickly became bounded by a plasma membrane with the centrosphere lying to one side in the cytoplasm. A few seconds later the centrosphere was seen to move against the margin of the hole and to produce a rounded bulge which occupied a considerable part of the hole.

The mitochondria in the fibroblast are slender, wavy filaments which undergo slow, sinuous movements among the sparsely-distributed fatty globules in the cytoplasm. It was found possible to touch individual mitochondria with the tip of a microneedle and to push them about without producing any visible effect, except possibly in causing them to appear more distinct. One long filament was even cut in two and each piece, as well as the neighbouring mitochondria, preserved their filamentous shapes.

On the other hand, a drastic tear of the cytoplasm or the destructive effect of puncturing the nucleus quickly causes the mitochondria throughout the dying cytoplasm to round up. The degenerative changes in the mitochondria, fig. 2, were observed to be as follows:—

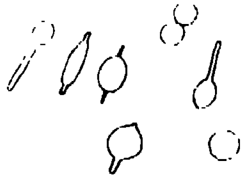

FIG. 2.—Swelling of the filamentous mitochondria following puncture of the nucleus and accompanying degenerative changes in the cytoplasm.

The filament at first swells either at one end or anywhere along the middle, and in some cases two or more swellings may occur on the one filament. The swellings then progressively enlarge and incorporate more and more of the filamentous portion until the entire mitochondrium has been converted into one or more delicately outlined sphærules. Under dark-ground illumination, these sphærules can be distinguished from the fatty globules by the much lower refractive index of their borders.

The nucleus is a deformable, fluid body which collapses when pricked. In the expanded cell it is a flattened, broad ovoid which reverts to a spherical shape when the body of the cell fills out by a release of its peripheral attachments. Within the optically empty interior are one or more vaguely-shaped nucleoli which can be pushed about and divided with a needle into smaller sphærules. The nucleus does not necessarily collapse until the needle has been removed.

Puncture of the nucleus of a mononucleated cell always results in the death of the cell. The immediate effect is a cessation of intra-cytoplasmic movements in the vicinity of the nucleus and a few seconds later throughout the entire cell. This is quickly followed by the appearance of a diffuse, very fine granulation probably identical with the "death granules" described by Lewis (1923), which produces a general opacity of the cytoplasm. During this time, the pseudopodia of the cell gradually retract. The nucleus becomes more and more opaque, and continues to shrink for as long as one to three minutes after being punctured.

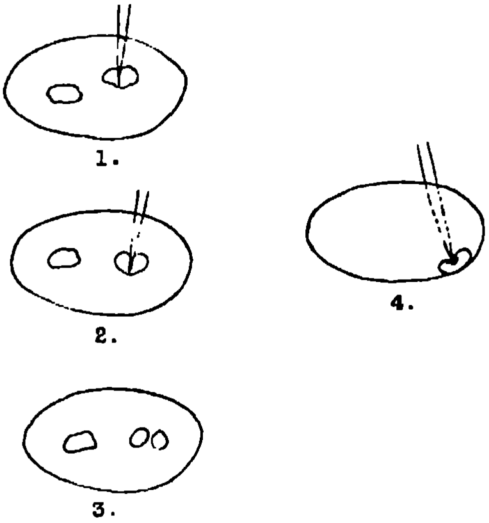

Fig. 3.—Manipulation of one of the nucleoli of a fibroblast: 1-3. dividing a nucleolus into two smaller sphærules: 4. pushing a nucleolus against the nuclear membrane.

A typical case of the death of a fibroblast, fig. 4, is recorded as follows:—

Within one minute after puncturing the nucleus the cell had shrunk considerably; two minutes later the nucleus was an irregular, crenated, granular body within a hyaline space. Brownian movement of the fat globules throughout the cell progressively increased and reached its maximum seven minutes after the cell had been punctured, while a definite membrane, about the liquefied cytoplasm, kept the oscillating granules from escaping. The membrane of the dead cell was then torn, upon which disintegration became complete and the internal granules escaped into the surrounding medium.

The nuclear remnant, as a coagulated body, was dragged out and stretched into a stringy, gelatinous mass. In another case in which the mitochondria were beautifully visible, the nucleus was punctured, and, after several minutes, the mitochondria degenerated into globules and a bubbling occurred at different regions on the surface of the cell. After 5 to 10 minutes, a restricted Brownian movement developed throughout the cytoplasm. The formation of bubbles on the surface of a dying cell is a peculiar phenomenon of the membrane undergoing degeneration. Some of the bubbles break away as delicate, hyaline vesicles and disappear in the medium. Tearing a degenerating cell occasionally accelerates the bubbling process. During this period the cell

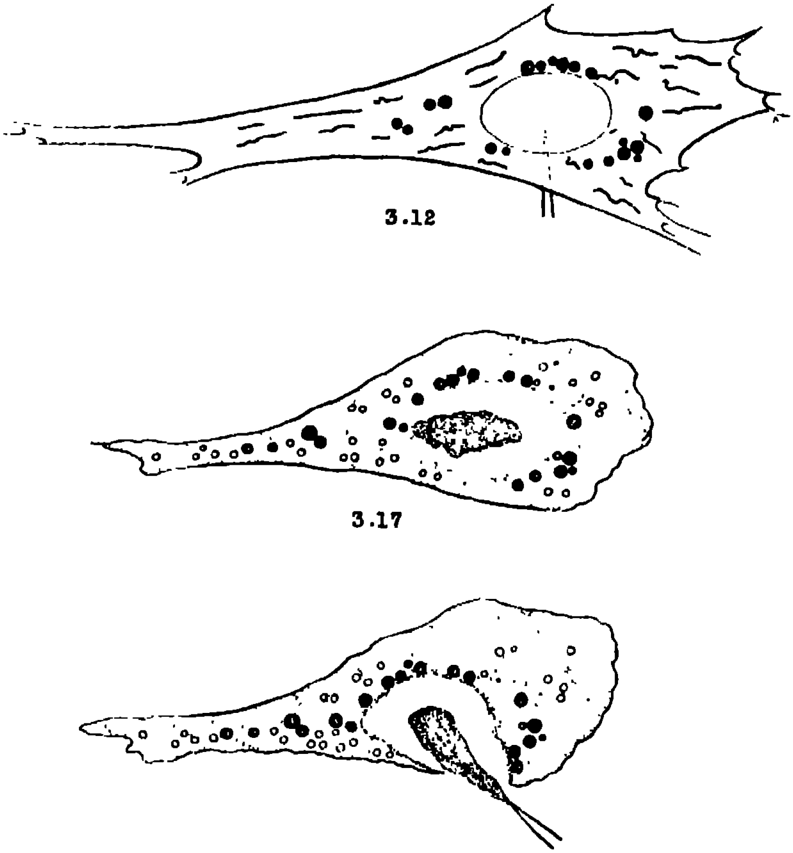

Fig. 4.—The effect of nuclear puncture on a fibroblast. The entire cell shrinks, the nucleus becomes crenated, a granular precipitate appears in both nucleus and cytoplasm and the filamentous mitochondria round up into spheres. The third drawing shows the nuclear remnant being drawn out of the coagulated cell by the needle.

may still remain adherent in places to the glass, and, by progressive retraction, leave behind long streamers of material which are in agitation and frequently round up into vesicles. The main substance of the granular cytoplasm finally shrinks into a coagulated mass.

In the case of binucleate cells, which are fairly common in slightly unhealthy cultures, damage done to one nucleus results in degenerative changes which may or may not involve the remaining nucleus. In several cases it was found that the presence of the other nucleus resulted in a complete recovery of the cell (Plate 36, fig. 1). A typical case is recorded as follows: In a well-spread-out fibroblast, one of two oval nuclei lying close together was slightly pricked with a sharply-pointed needle. A pseudopodium extending from the vicinity of the injured nucleus was completely withdrawn and the flow of its cytoplasm into the main body of the cell coincided with the collapse of that side of the pricked nucleus towards which the flow was directed. The cytoplasm in the vicinity became clouded with the appearance of fine, diffuse granules. A minute later another pseudopodium extending from this region was withdrawn and a general movement became apparent which carried fat bodies and mitochondria into the region of the injured nucleus which shrank still further into a small, wrinkled mass. The other nucleus gave no sign of degenerating. Five minutes later, pseudopodia were extending again from the region which had been affected, the cloudiness disappeared and, within ten minutes after the puncture of the one nucleus, the cell was again completely expanded and had regained its former appearance, except in having been converted from a binucleate into a mononucleate cell.

In another case one of two nuclei was punctured in a cell in which the two nuclei were widely separated. Localised retraction of the cell occurred and the fine granules appeared within a minute. Three minutes later the cytoplasm was clear again, and six minutes after the puncture the cell had completely expanded and appeared normal. The second nucleus was then punctured, whereupon death granules appeared all through the cell which soon shrivelled into a degenerated mass.

Fibroblasts sometimes occur in groups in which the contiguous cell-boundaries are invisible. A case in point was a structure which appeared to be a large binucleate fibroblast. One nucleus was punctured and the result was that the death changes about the injured nucleus and the consequent shrinkage brought into view the outline of the contiguous cell which remained unaffected.

2. *Epithelial Cells of the Intestine.*—The sheets of epithelium which outwander in these cultures make beautiful objects under dark-ground illumination. The

cell-boundaries are invisible, but the area of each cell is indicated by the optically empty, broad ovoid nucleus around which are clustered filamentous mitochondria which show up as pale white, wavy lines in constantly shimmering movement. Here and there among the mitochondria is an occasional highly refractive fat-like globule. In some of the nuclei can be seen one or two faintly outlined nucleoli.

A culture (Plate 37, fig. 2) containing a beautiful sheet of epithelium with cells full of filamentous mitochondria, was mounted on the microscope stage and one of the nuclei in the middle of the sheet was punctured. The mitochondria around the injured nucleus stopped moving, became much more distinct than before, and the outline of the cell became apparent as its border, except at two opposite ends, gradually shrank away from that of the neighbouring cells. Within two minutes the mitochondria in the affected cell were distinctly thicker and shorter than before, while the surrounding cells maintained their normal appearance and position. One minute later (three minutes after the puncture) the nucleus became whitish owing to the appearance of fine granules and finally shrank into a coarsely granular body with a distinct outline. The mitochondria, in the meantime, became prominently doubly contoured and developed localised swellings so as to appear beaded. Within another minute all the mitochondria were converted into sphærules with pale white contours less refractive than the highly glistening fat-like globules in the cell. Six minutes after the nuclear puncture the cell was a shrunken coagulated, granular mass, held in place only by two opposite tapering ends and with a broad gap on either side separating it from its neighbours.

There was no evidence of transmission of injury from cell to cell, and the two persisting points of attachment of the shrunken cell recall a similar phenomenon already described in the microdissection of cells of peritoneal mesothelium (1925).

The ovoid form of the nucleus in the living cells of this epithelium is due to the stretched condition of the cells, for the nucleus changes to a spherical shape when the tension is released by loosening the cell from its attachments.

3. *Pigmented Epithelial Cells of the Retina.*—These cells are among the most beautiful objects under dark-ground illumination. The epithelium grows as a continuous sheet of very much flattened, polygonal cells, the boundaries of which are invisible. Each cell has its broad, ovate nucleus which is optically empty except for one or two vaguely shimmering nucleoli. The rest of the cell is filled with golden-hued, glistening pigment rodlets which are at one moment stationary and at another scurrying about in groups and singly along

various paths through the cytoplasm but always in the plane of their longitudinal axes. The rodlets always keep within their respective cells and it is this fact which enables one to distinguish the boundaries of the cells in what otherwise appears to be a continuous sheet.

The cells of a sheet are attached only along their borders to the neighbouring cells and, at the periphery of the sheet, to the glass of the coverslip. When, therefore, the border cells become detached from the glass the sheet contracts as a whole.

A broad cytoplasmic process, extending into the medium from a cell on the periphery of the sheet, was carefully cut off by drawing the tip of a needle

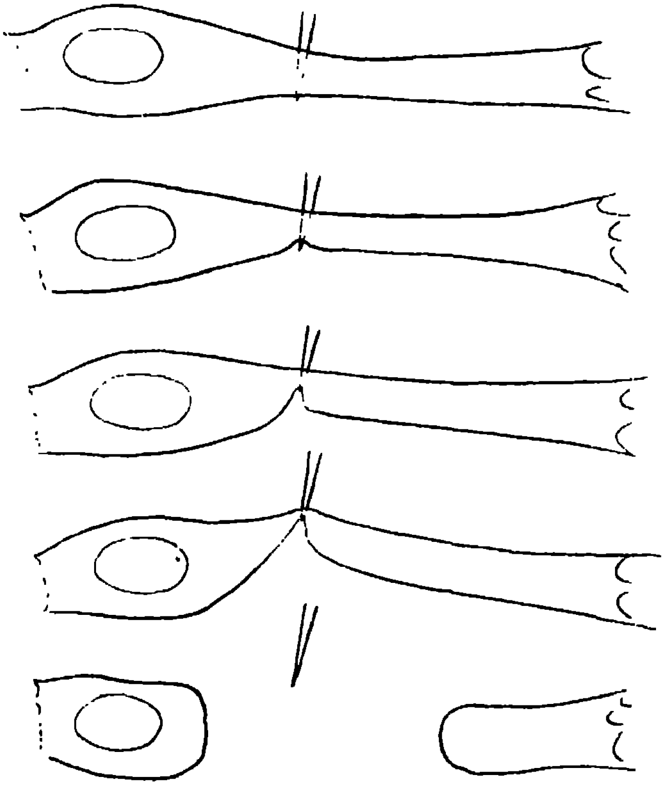

FIG. 5.—Elongated pigment epithelial cell showing the effect of cutting off a cytoplasmic process. Note the elasticity of the cell as indicated by the retraction of the two parts away from the cut towards the two attached ends.

slowly across its middle (text-fig. 5). The pull of the two ends of the cell spread the borders of the cut as the needle passed from one side to the other. The separation was completed without causing the original attachments to be loosened and the two parts of the cell shrank back to their respective ends, piling up the contained pigment rodlets. In such an operation the nucleated fragment was frequently seen to resume internal movement and to expand again.

In five out of seven cutting experiments there was no recovery of movement in the non-nucleated fragment which soon degenerated by developing Brownian movement of its granules or by coagulating. One of the two remaining cases recovered after five minutes only to the extent of slowly extending a short pseudopodium from the cut surface. The last case demonstrated without any doubt that a non-nucleated fragment is capable of resuming internal movement and of expanding by forming pseudopodia. Because of its significance this is recorded in detail as follows.

An elongated cell with the nucleus at the proximal end and with active internal movement, was carefully cut across close to the nucleus. The long non-nucleated portion shrank back to its attached end. The pigment rodlets, at first in a crowded heap and motionless, soon recommenced their individual movements and the cytoplasmic fragment expanded, with the rodlets scurrying about in a manner typical of their normal movements. This non-nucleated fragment was watched for fifteen minutes and it was active during all this time.

The degree of injury induced by tearing the cytoplasm alone depends upon the extent of the tear. In a much expanded cell with the cytoplasm reduced to a thin film, a puncture in the cytoplasm produced a hole which increased in size as the surrounding cytoplasm receded. Intracellular migratory movement of the granules and pigment rodlets ceased for some time. Upon its resumption, the hole steadily diminished and disappeared. On the other hand, when the cytoplasm is injured by a tearing action of the needle, death generally ensues.

The extremely flattened form of the cells make it difficult to puncture them without causing the tip of the microneedle to pass completely through the cell. The stretched condition of the cell generally causes the cytoplasm around the site of the puncture to retract and to leave a space which develops a boundary about a hole. A similar effect is frequently seen when the nucleus is punctured. The peripheral pull of the stretched cell may persist for some minutes before cytolysis is completed with the result that a perinuclear space develops, not only from the shrinkage of the injured nucleus but also from the retraction of the surrounding cytoplasm.

A striking instance of this occurred in a long extended cell, the two ends of which were attached. The nuclear puncture did not cause a release of the attached ends. The result was that the hole, which developed in the middle of the cell where the nucleus had been, steadily enlarged until, four minutes after injury had been initiated, the stretched coagulum was so weakened in the region of the hole that the mass parted in two and the two portions then contracted toward the attached end. In common with the other types of cells studied all internal movement immediately stops when the nucleus is punctured. Coagulation granules then appear in the cytoplasm and the cell slowly shrinks away from its neighbours leaving the latter unaffected (Plate 37, fig. 3).

In the two cases in which one nucleus of a binucleate cell was punctured, the other nucleus also underwent degeneration and the cell died.

4. *Macrophages.*—The macrophages are the most actively moving cells observed in the cultures. They occur in cultures of any tissue, but are more numerous in some than in others. Their mode of attachment in the liquid culture medium differs from that of the other cells investigated in that most if not all of the surface exposed to the glass of the coverslip is adherent. In other cells this adhesion seems to be restricted to the peripheries of their extensions.

Another peculiarity of the macrophage is its reaction to probing with a microneedle. Other cells quickly or slowly retract their expansions and shrink away from the glass of the coverslip to which they are attached. The macrophage, on the other hand, tends to react to manipulation by expanding still more than before. In a typical case a broad lamelliform expansion on the coverslip was pushed in at one place by repeated probing until a deep bay resulted along the border of the expansion (text-fig. 6). The needle was then removed and within a few seconds small lobular pseudopodia began to form at the bottom of the bay. The lobules, by filling out and fusing with one another, carried the contour of the cell outward until the bay was obliterated.

Frequently, mechanical stimulation causes an extended hyaline pseudopodium to be withdrawn, but this is generally soon followed by the extension of another.

The macrophage also differs from the other cells in its reaction to nuclear puncture. Instead of undergoing shrinkage the cell expands while the centrally located granular cytoplasm tends to shrink and by so doing accentuates the effect of the expansion of the hyaline periphery.

Sometimes puncturing the nucleus is followed, a minute later, by the appearance of a pronounced Brownian movement of granules in several areas throughout the cytoplasm. The intensity of the Brownian movement increases and the surface membrane may throw out hyaline, bubbly protrusions which fuse with one another, recede and extend again. The outline of the membrane stands out distinctly and the rapidly oscillating granules frequently bump against it without adhering. The membrane of these dying cells is very adhesive to glass and can be pulled out in viscid strings on the end of a needle.

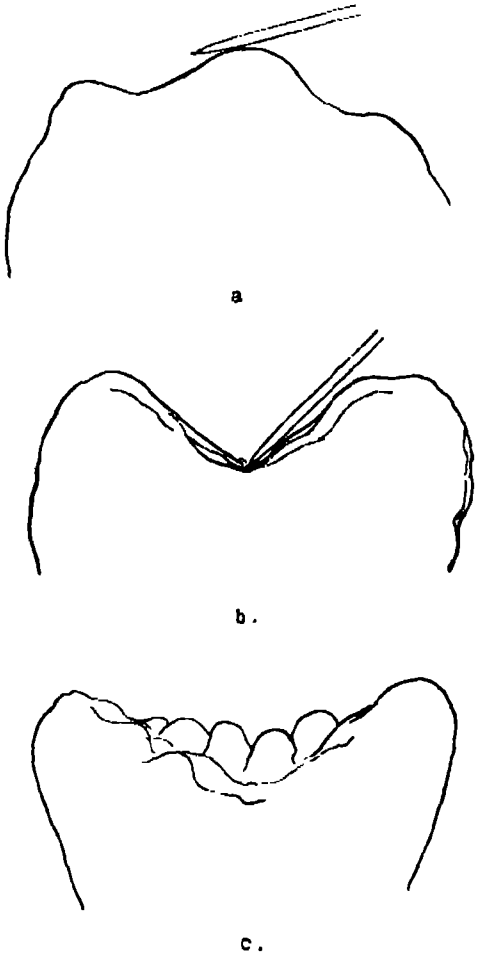

FIG. 6.—Part of the periphery of a macrophage showing the effect of push in a lamelliform expansion of the cytoplasm. (*a*) Expansion about to be pushed in. (*b*) Expansion pushed in and bay formed in cytoplasm. (*c*) The formation of lobular pseudopodia at the bottom of the bay after removal of the needle.

When a protrusion, containing granules in Brownian movement, withdraws into the body of the cell the movement slows down and stops as the bulging protrusion is withdrawn. In one recorded case the above changes continued for ten minutes after which the cell was torn to pieces with needles. One protrusion was torn open and the contained granules were liberated in the surrounding medium where they continued their oscillatory movements. Elsewhere the cell had flattened considerably, no movement of the granules was visible and the previously prominent outline of the membrane disappeared. The entire mass of the cell remnant was now of a consistency easily torn with needles into viscid, jelly-like, semi-elastic strands.

In searching for a binucleate macrophage, several times what appeared to be one turned out to be two contiguous mononucleate cells. When one nucleus was punctured the characteristic death changes took place in the one cell while the other remained intact.

Finally, a binucleate cell was encountered and one of the nuclei was punctured. Within three minutes the punctured nucleus became pronouncedly granular and swelled in size. The cell as a whole expanded enormously and the second nucleus also granulated. Four minutes after this the cell outline began to wrinkle and the peripheral hyaline zones became clouded with the appearance of fine granules. Within ten minutes after the puncture of the one nucleus the cell had been converted into a loose coagulum containing the remains of the two nuclei as two prominent, granular spheres, the punctured one twice the size of the other. The nuclear remnants, on being pulled out into the medium, lost their opacity and persisted for some time as jellied, hyaline bodies.

A noteworthy phenomenon is the behaviour of phagocytes in the vicinity of any cell which has been injured with needles. The phagocytes move to the injured cell and cluster on it. Indeed, in the tearing experiments on macrophages, the neighbouring phagocytes proved to be a decided nuisance by getting in the way of the needles during operations.

When long, cylindroid myoblasts were tórn a localised area of injury could be produced, and the phagocytes were seen to be attracted only to the injured region.

5. *Skeletal Myoblast.*—The myoblasts were mostly from two- and three-day cultures obtained from the limbs of nine-day chick embryos. Most of the myoblasts were long cylinders and averaged about 6 micra in width and 70 micra in length, and extended from the explant at various angles among fibroblasts and macrophages.

In the liquid medium in which they were cultured the myoblasts appeared to be attached only by their two ends. The proximal end is usually firmly embedded in the explant and the peripheral end is spread out as a thin sheet stuck to the surface of the coverslip. When one end is detached the fibre shortens and thickens, but maintains its elongated, cylindroid shape.

Under dark-ground illumination the cytoplasm appears to be filled with slender mitochondria more or less longitudinally arranged and mainly in the peripheral layer of the cytoplasm. The peripheral arrangement is best developed in the larger fibres. Numerous ovoid nuclei are disposed in groups along the central region. The fluid cytoplasm containing the mitochondria and nuclei is enclosed within a membrane of a toughness considerably greater than any cell-membrane so far encountered in cells of tissue cultures.

With a lateral push of a needle the myoblasts could be bent and stretched considerably without detaching their ends. When released, they immediately returned to their original form, indicating a pronounced degree of elasticity.

The myoblast was found to be surprisingly tough and difficult to tear. This is in marked contrast to the ease with which the long extended pseudopodia of fibroblasts or of pigment epithelial cells could be cut off.

When the surface of a myoblast, in its normal stretched condition, is torn, the region about the tear swells and the interior wells out as a jellied mass. The torn membrane undergoes degeneration by forming bubbly excrescences which soon break down, and the mitochondria granulate. The degeneration tends to spread in both directions from the site of injury. During the swelling the nuclei in the affected region become distinct as granular sphærules and collect in numbers in the bulging area. The dead, coagulated nuclei could be seized with needles and stretched.

It is possible to cut completely through a myoblast by pressing on it with the side of a cylindrical needle. The cut ends round off with little loss of the internal cytoplasm, and the two parts of the fibre then retract considerably. In several cases, one of the nuclei of an uninjured myoblast was punctured with the tip of a fine needle. This caused the cylindroid myoblast to undergo a pronounced and momentary contraction, followed by a slow and progressive degeneration.

Numerous fibres were punctured with the tip of a fine needle. In some cultures a considerable number of fibres on the same slide responded readily to mechanical stimulation. These fibres varied in size. The response could be repeated several times on the same fibre, but between the responses there was always a refractory period of about ten seconds or more, during which pricking or even tearing the fibre called forth no twitching response.

The twitch which occurs on pricking is a sudden, slight contraction and thickening of the fibre, followed by a relaxation. Occasionally, the contraction and thickening is most pronounced about the pricked region, and the following relaxation is slower, but usually both reactions are too quick to enable the eye to appreciate any localised effect.

6. *Cells (fibroblastic) in Mitosis.*—An early telophase cell can be seized by one pole and moved about in the fluid medium without loss of shape, suggesting a pronounced stiffness of the cell as a whole.

In a telophase cell with normally bubbling cytoplasm the cytoplasm and the bubbling protrusions can be pierced with impunity. If, however, the surface is torn, all movements stop at once and the cell dies.

In one case a considerable portion of the bubbling cytoplasm at one pole was cut off. The main body of the cell ceased movement, but the cut-off portion continued to bubble for a minute or so. The continuance of the bubbling process when the cell is probed or punctured and the ease with which internal Brownian movement is set up when the surface of the cell is torn are reminiscent of the behaviour of the soft-bodied macrophages to the needle.

Transmission of injury from one daughter cell to the other was demonstrated in a late telophase in which the two daughter cells were connected only by a very slender stalk. One cell was punctured, the bubbling increased, but within a few seconds the cytoplasmic granules became distinct and movement stopped. The other daughter cell continued its bubbling movements for another minute, when it in turn ceased moving and its granules became distinct as it died.

General Results.

The consistency of the protoplasm of the different types of cells investigated was found to vary, being more fluid in some than in others. All the cells, however, give the appearance of a considerable degree of elasticity. Thus, if a cell is stretched by two needles it can be made to elongate greatly, and when the tension is relaxed the cell tends to retract and thicken. With the possible exception of the macrophage, cells growing on a coverslip are in a state of considerable tension during life; they are attached to the coverslip only by the tips of their pseudopodia which, if detached from the glass, more or less rapidly retract into the main body of the cell. Similarly, if the cytoplasm of an elongated cell is cut in half transversely, the two halves retract from the cut.

There is evidence that the cytoplasm is enclosed in a morphologically definite membrane. If a cell is probed with a needle, the surface becomes indented, and when the needle penetrates the cytoplasm it does so suddenly, as if it had passed through a resistant membrane into a softer interior.

The consistency of the cell membrane varies considerably in the different types of cells. The stiffest encountered was that of the myoblasts, and the softest and most pliable were those of fibroblasts undergoing mitosis and of the macrophages.

A peculiarity of the membrane of the last two mentioned cells is the ease with which it is thrown into rounded elevations, which repeatedly rise and sink, so as to give the appearance of a recurrent bubbling of the surface. The membrane, under such circumstances, appears to be extremely soft, but still offers a certain degree of resistance to probing with the needle.

Although the interior of the cytoplasm seems to be softer than its enveloping membrane, it is probably, at least in most cells, somewhat viscid, for movements of the needle within the cell frequently create a disturbance of the intracellular bodies some distance away. The semi-fluid nature of the cytoplasm is further suggested by the fact that the granules and small fat globules within the cell do not show typical Brownian movement, but only a faint oscillation, although, if liberated into the fluid medium by tearing the cytoplasm, they immediately show violent Brownian motion.

From numerous observations, the impression is gained that the pronounced elasticity of the cells is due more to the membrane than to the internal cytoplasm.

The nucleus also appears to possess a definite membrane enclosing a fluid or semi-fluid interior. The nuclear surface can be indented by the needle, and is sometimes difficult to puncture, as the nucleus tends to elude the needle-point, behaving like a slippery sac. When once the needle has penetrated, the nucleus slowly collapses into a shrivelled mass, leaving around it a non-granular space in the cytoplasm more or less representing the boundary of the original nuclear area.

The deformability of the nucleus is shown by the fact that the ovoid shape, which it possesses in a well-spread-out cell, readily changes to that of a sphere when the tension is released by loosening the cell from its attachments.

In agreement with previous work (Péterfi and Olivo, 1925, and Chambers and Rényi, 1925), the nucleus was found to be very sensitive to injury, and in every case in which the nucleus is even slightly punctured, the cell sooner or later dies.

A frequent peculiarity of degenerating macrophages, also noted by Péterfi

(1927), is a considerable expansion of the cell and a bubbling of its surface. The bubbling reaction was observed also in degenerating fibroblasts.

The result obtained by puncturing one nucleus in a binucleate cell was very interesting. As described above, immediately after puncture, the injured nucleus begins to crumple and become opaque, death-granules appear in the surrounding cytoplasm, intracellular movement stops in that region and the pseudopodia nearest the affected nucleus rapidly retract into the main body of the cell, while the uninjured nucleus and the cytoplasm in its vicinity remain normal. After a few minutes the cell, instead of degenerating as in the case of the mononucleate cell after nuclear puncture, begins to recover and, except for the presence of the shrivelled punctured nucleus, appears normal in structure and behaviour. Such recovery was not observed in all the binucleate cells subjected to a single nuclear puncture, for in some cases the second nucleus also becomes affected and the entire cell degenerates.

In agreement with previous work (Péterfi and Olivo, 1925, and Chambers, 1924), it was found that the cytoplasm is much less susceptible than the nucleus to permanent injury. In their usual state, the cells are expanded and lie flat against the coverslip. If a small puncture is made in the cytoplasm, the needle passes completely through the cell and a hole results. Intracellular movement instantly stops in the immediate neighbourhood of the puncture, but after a few minutes the hole gradually closes and intracellular movement recommences.

A drastic tear of the cytoplasm may result in death of the cell (*cf.* Levi, 1926). When this occurs, the first sign is a spread of degeneration of the cell-membrane. The rest of the sequence of events is similar to that following nuclear puncture except that the collapse of the nucleus is considerably delayed or may not occur and, instead, there may be a certain degree of swelling. It is of interest to note that the nucleus tends to become hyaline when it is dragged out of the cell, where it can be stretched and torn like a gelatinous body.

A peculiar condition of the extended and spread out cells in a culture is the considerable tension under which they appear to exist. Under this tension the nucleus is flattened and the cytoplasm is reduced, in places, to an extremely thin sheet. A hole produced by a severe tearing puncture in such a sheet progressively increases in size as the material of the cytoplasm recedes. In a sheet which is longer than it is broad, *e.g.*, in an extended pseudopodium, the enlarging hole may soon reach the lateral borders of the sheet which finally breaks through, thus separating the main body of the cell from its peripheral portion. The cytoplasm along the border of the hole develops a membrane which is extensible. In some cases the formation of the membrane is not quick enough to prevent the disintegration and escape of cytoplasmic granules into the fluid filling the hole. Then the granules exhibit Brownian movement.

In confirmation of the observations of Olivo (1925), it was found that a comparatively large portion of the cytoplasm can be completely amputated without causing the death of the nucleated portion of the cell. In such experiments on cells spread out on the glass, both parts of the cell shrink back from the needle as soon as the incision is made. The nucleated fragment may recover completely, so that new pseudopodia are protruded and intracellular movement is renewed. In addition to this, it was also found that the non-nucleated fragment may show at least temporary recovery by a resumption of both internal and pseudopodial movement for as long as fifteen minutes after the operation. Levi (1926) has described similar survival, and persistence of amœboid movement, in the case of fragments of nerve fibres cut off from the parent cell by micro-manipulation.

Experiments in which individual cells are killed by operation demonstrate very clearly the important fact already pointed out for fibroblasts (Chambers, 1924) and for various epithelia (Kredel, 1927) that, in the different cell-types investigated, each cell is structurally independent of its neighbours. This was found to be true even in cases where the cells were in such intimate contact that no cytoplasmic boundaries could be distinguished. Thus, if the nucleus of a cell forming part of a sheet of epithelium is punctured, the cell degenerates as described above. In the course of this degeneration the cytoplasm shrinks from the surrounding cells which remain completely unaffected, showing no degenerative changes or even a temporary cessation of their normal, intracellular movement.

The only case in which transmission of the effect of injury was observed was in a very late telophase stage in which the two daughter cells had begun to move apart, but were still connected by an attenuated stalk. The degeneration of the punctured cell was followed within one minute by the degeneration of the sister cell.

Our inability to find any evidence of protoplasmic continuity between cells in single layered sheets is corroborative of previous work on this point (Chambers and Rényi, 1925, and Kredel, 1927). Levi (1923) describes a few exceptional cases in which he observed what he considered to be an actual passage of chondrioconts along protoplasmic extensions from one cell into another in fibroblasts and myoblasts. In pigment epithelial cells we have several times observed what appeared to be a similar phenomenon with the pigment rodlets in the cytoplasm. However, by moving the cells about and by watching the spread of degeneration after injuring one of the cells, we were convinced that the apparent passage of material from cell to cell was in reality only an optical effect due to the superposition of the extended pseudopodia of one cell over the body of the other.

The reaction of the cells to irritation by probing with microneedles brought out some points of interest. With the exception of skeletal myoblasts and of macrophages the reaction usually is a slow and gradual detachment from the coverslip and a retraction of the extended pseudopodia. The macrophage is peculiar in its reaction by spreading out instead of retracting. For example, when prodded it tends to throw out broad lobate pseudopodia which partially envelop the needle. The elongated, cylindroid, skeletal myoblasts react by a sudden contraction followed by a relatively slow relaxation. The myoblasts which give such typical twitching reactions are devoid of visible cross-striations and of typical myofibrillæ. Rényi and Hogue (1931) report obtaining by mechanical stimulation a quick shortening of outgrowing muscle buds in cultures of skeletal muscle from chick embryos 4–10 days old in Locke-Lewis medium. A typical relaxation of the buds was not observed. Spontaneous rhythmical contractions described by Lewis (1915, 1920), and Friedheim (1931), were not observed in the few cultures that we used.

A frequently observed phenomenon with reference to macrophages was their positive reaction at a distance to the presence of cells undergoing cytolysis from mechanical injury. This was particularly noticed in cultures containing numerous macrophages. As soon as a cell had been torn with needles, be it a myoblast or another macrophage, several macrophages, lying in the field of an oil immersion objective, migrated toward and came into close contact with the injured cell within one or two minutes after the injury.

Summary.

1. A variety of cells in tissue culture in a liquid medium obtained from a mixture of embryonic juice and plasma were operated upon under dark-ground illumination.

2. In all the cells studied prodding with the needle indicated the existence of a membrane which is stiffer than the enclosed cytoplasm. The elasticity of the cells seems to be a function primarily of the membrane. The nucleus is a

deformable, ovoid body with a morphologically definite membrane and a fluid interior containing no visible structural elements except one or two nucleoli.

3. In agreement with previous work on other cells, these cells are not appreciably affected by repeated puncturing of the cytoplasm. Cytoplasmic granules can be pushed about, and mitochondria can be cut in two without any visible degenerative changes. However, a drastic and quick tear of the cell membrane causes either a localised or general degeneration of the cell.

4. The nucleolus in a living nucleus can be divided in two with a microneedle, but the nucleus does not long survive the effect of the puncture.

5. In agreement with previous work on ova and somatic cells, the nucleus was found to be very susceptible to mechanical injury, and when the nucleus shows signs of degenerating the effect is transmitted to the surrounding cytoplasm. In cells in tissue culture, as Péterfi also found, the merest puncture of the nucleus always resulted ultimately in degeneration. In a mononucleate cell the degeneration gradually spreads until the entire cell is involved. In a binucleate cell, puncture of one nucleus results in degenerative changes which frequently are restricted to the vicinity of the injured nucleus. In some cases the degeneration spreads to the other nucleus and through the entire cell. In several cases, however, there occurred the significant reaction which demonstrates the effect of the presence of a second nucleus in causing a reversal of the degenerative changes. After several minutes, these visible signs of degeneration in the cytoplasm disappear, the cell extends pseudopodia afresh, and assumes a typically normal mononucleate appearance, except for the presence in the cytoplasm of a diminutive and crumpled, gelatinous remnant of the injured nucleus.

6. The signs of cellular degeneration following cytoplasmic tearing or nuclear puncture are: (1) retraction of pseudopodia and a gradual detachment of the cell from surfaces to which it had been adhering. In the case of macrophages, this is preceded by a considerable expansion of the cell and a peculiar bubbling of its surface; (2) coagulation of the nucleus, preceded, in the case of nuclear puncture, by a partial collapse of the nucleus; (3) the appearance of a fine, diffuse granulation of the cytoplasm; (4) conversion of the slender mitochondria, if present, into sphærules; (5) the appearance of active Brownian movement in localised regions or throughout the interior of the cell; (6) finally, a general shrinkage and collapse of the cell into an amorphous coagulated mass.

The surface membrane of the degenerated cell appears to be a structure quite different from the normal membrane of the living cell. The living cell membrane undergoes degeneration by a breaking down of its uniform contour.

In the case of degeneration subsequent to tearing, the breakdown of the membrane spreads from the site of the injury.

7. The centrosphere in the fibroblasts probably is of a consistency greater than that of the surrounding cytoplasm. It can be punctured without change in its usual appearance.

8. Long cylindroid, multinucleated, skeletal myoblasts in cultures derived from the limb of a nine-day chick, can be made to twitch repeatedly with a refractory period between successive twitches.

9. Mitotic cells of fibroblasts are very susceptible to injury. Puncturing protrusions causes no injury, but a slight tearing action kills the cells.

10. With the possible exception of macrophages, all the cells studied are attached either along their edges to neighbouring cells or along the tips of their extended pseudopodia.

When one cell in a sheet of epithelium is injured, the cell shrinks from its neighbours, except possibly at a few points.

11. The effect of injury was never seen to be transmitted from the injured cells to its neighbours. The only case of such transmission occurred from one daughter cell to the other during the period while the two were still connected by an attenuated protoplasmic stalk.

12. Macrophages are attracted from a distance to a cell which has been recently caused to degenerate through mechanical injury.

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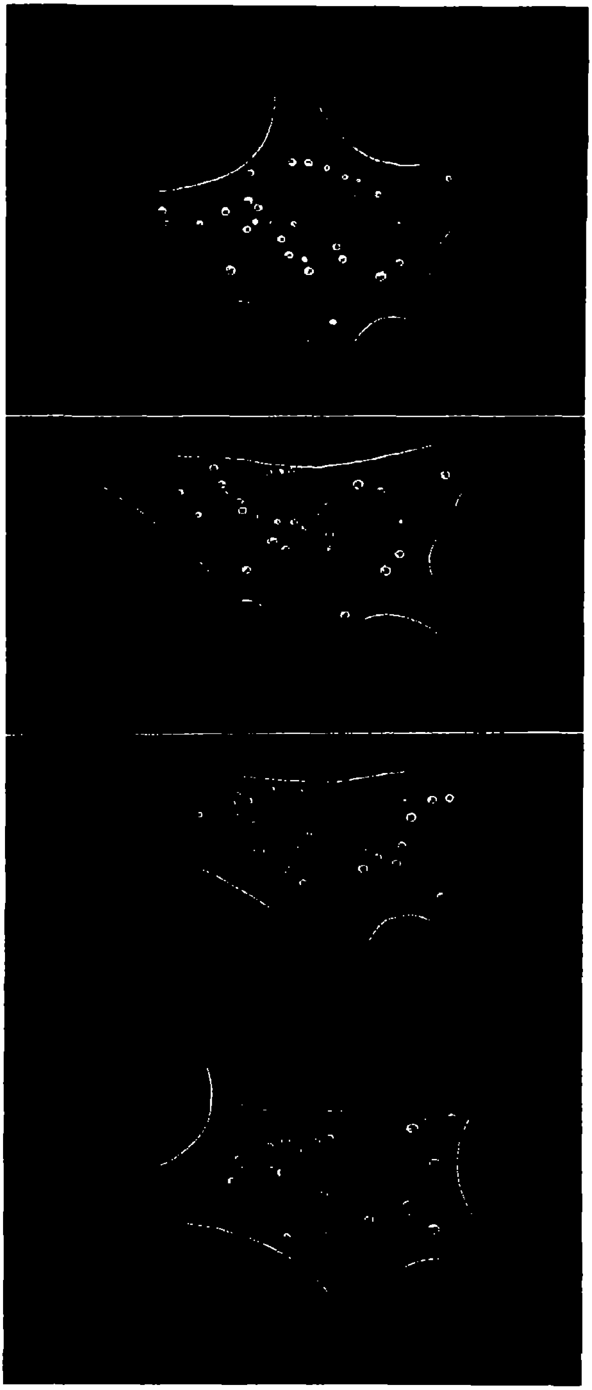

Fig. 1. (H.B.F. *del.*)

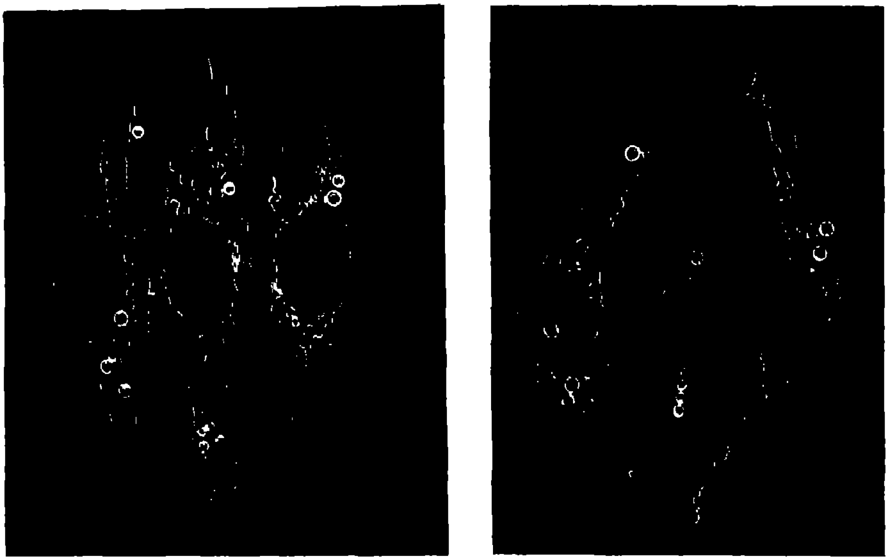

FIG. 2.

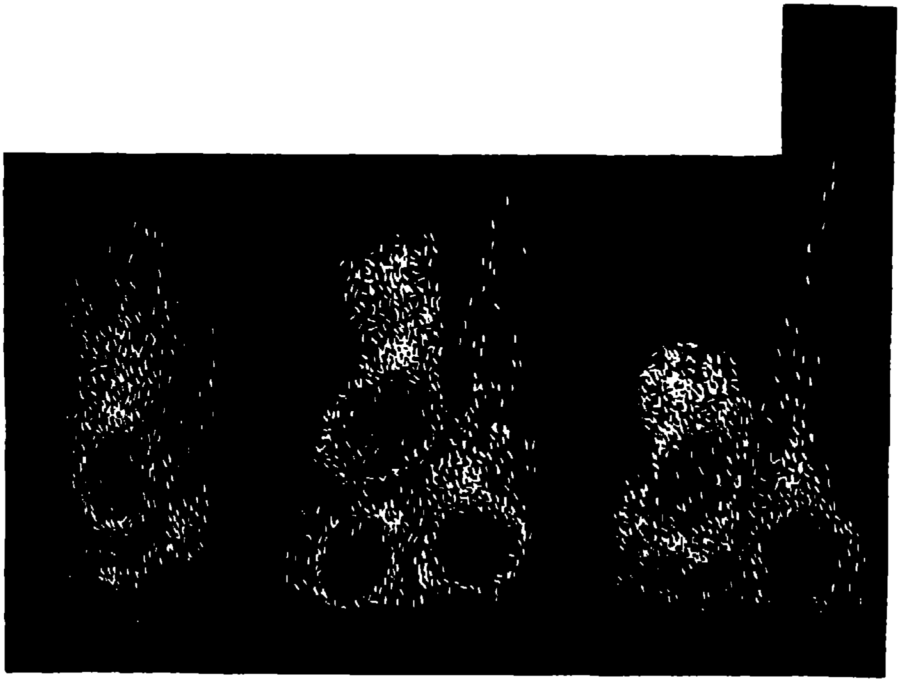

FIG. 3.

(H B F *del.*)

DESCRIPTION OF PLATES.

PLATE 36.

FIG. 1.—The effect of puncturing one nucleus of a binucleate fibroblast. (*a*) Cell immediately before puncture. (*b*) Cell immediately after puncture. The punctured nucleus has collapsed and become opaque and the adjacent pseudopodium has been completely withdrawn; the cytoplasm around the injured nucleus has become clouded with fine diffuse granules. (*c*) Cell four minutes after puncture. Another pseudopodium has been partially withdrawn, the punctured nucleus is still more shrunken and has been carried partly over the uninjured nucleus which is quite unaffected. The granulation of the cytoplasm around the punctured nucleus is beginning to disappear. (*d*) Cell seventeen minutes after puncture. New pseudopodia have been protruded and the cell is again completely expanded and except that it is now mononucleate, has regained its former appearance. Note the crumpled remnants of the punctured nucleus.

PLATE 37.

FIG. 2.—(*a*) Three cells in a sheet of intestinal epithelium. The cell boundaries are invisible. The nucleus of the middle cell is about to be punctured. (*b*) Same cells eight minutes after puncturing the nucleus of the middle cell. The cytoplasm of the injured cell has contracted away from that of its two neighbours from which it is now separated by a broad gap on either side and is only attached by its tapering ends. Note the shrinkage and granulation of the punctured nucleus, the granulation of the cytoplasm and the beading of the mitochondria in the injured cell. The two neighbouring cells appear perfectly normal.

FIG. 3.—The effect of nuclear puncture on a pigment epithelial cell. (*a*) Immediately before puncture. No intercellular boundaries are distinguishable. (*b*) Ten minutes after puncture. The outline of the injured cell is now distinguishable and both nucleus and cytoplasm have shrunk. Note the granulation of the nucleus. The neighbouring cells are unaffected. (*c*) Thirty minutes after puncture. The shrinkage of nucleus and cytoplasm is still more pronounced in the injured cell but the neighbouring cells remain normal.

Address of the President, Sir Frederick Gowland Hopkins, at the Anniversary Meeting, November 30, 1931.

It is right that at our Anniversary Meeting we should have in mind the losses that our Fellowship has suffered during the year that has just passed. We have to deplore to-day the deaths of no less than four of our distinguished Foreign Members, together with fourteen Fellows of the Society.

ALBERT AUGUSTE TOUSSAINT BRACHET, of Brussels, was a distinguished leader in the science of embryology. He was one of the pupils of van Beneden and carried on traditions derived from that master of the subject, though on lines of his own. His earlier work dealt chiefly with the morphological facts of development, but he later made important contributions to experimental embryology. His researches were specially concerned with the early stages of development in the amphibia, and his work threw important light upon the problem of localisation in the developing egg. His later interests and contributions were concerned with what may be described as the physiological factors and conditions which initiate development. He was elected as a Foreign Member of the Royal Society in 1928.

MARTINUS WILLEM BEIJERINCK. The experimental researches of this distinguished Dutch biologist covered an extraordinarily wide field; they broke much ground that was new, and almost always led to results of high importance. He is best known, perhaps, for his discovery that the root nodules of papilionaceous plants are caused by an organism which he succeeded in cultivating outside the nodules and called *Bacillus radicicola*. This discovery, and Beijerinck's own further work on the subject, were of fundamental importance in demonstrating and explaining the biological fixation of nitrogen. His researches on other aspects of bacteriological metabolism were also of the greatest interest: his studies, for instance, of luminous bacteria, of the oligonitrophilous and oligocarbophilous organisms; of an organism which, while liberating nitrogen from nitrates, obtains its energy by oxidising sulphur. This is but one example of many investigations all tending to illustrate remarkable diversities in the metabolism of living cells. His work on the mosaic disease of the tobacco plant first introduced a knowledge of viruses into

Western Europe. Beijerinck was a learned botanist, and almost as well qualified as a chemist. Much of his work indeed was done in fields other than those described. It was always illuminating. He became a Foreign Member of this Society in 1926.

ALBERT ABRAHAM MICHELSON. This highly-gifted physicist is doubtless best known for his share in the classical Michelson-Morley experiments contrived to detect any possible effects of the earth's rotation upon the velocity of light. In his younger days Michelson was instructor in physics and chemistry under Admiral Sampson at the American Naval Academy. Later he studied in Berlin and Heidelberg, and also in Paris. On his return to the United States in 1883 he held chairs at Cleveland, Ohio, and at the Clark University, and finally in 1892 went to the Chair of Physics at Chicago. In 1925 he was appointed to the first of the Distinguished Service Professorships instituted by the last-mentioned University. It was in Optics that Michelson always excelled, and he seems to have been from the first attracted to the study of the interference of light, a preference on his part which proved fortunate for physical science. In the use of the interferometer he was a magician. In 1890 he first suggested that interference methods might be applied to astronomical problems. His grasp of this technique and his sense of its possibilities led to great results, one of which may receive special mention. Eddington had made the prediction, for adequate reasons, that a star like Betelgeuse must have the dimensions of a solar system, and by using data available from his own theory of stellar constitution he made an estimate of the probable diameter of that star. To the direct measure of the angular dimensions of Betelgeuse Michelson's interferometer was now applied. The capacity of the instrument proved equal to expectations and the observers realised that for the first time they had been able to measure the angular dimensions of a star. An estimate of its distance gave the actual diameter and Eddington's estimate was confirmed. Interferometric methods have doubtless yet many and important applications before them. For the study of atomic radiations Michelson designed a very successful instrument, the Echelon Spectroscope. The famous Michelson-Morley experiment called, of course, for the utmost refinement of measurement, and in that art Michelson was supreme. The result formed, as we know, the very basis of Relativity. Michelson became one of the Foreign Members of this Society in 1902, and was awarded our Copley Medal in 1907.

Baron KITASATO. Kitasato was one of the greatest among bacteriologists. So far back as 1889, his success in obtaining for the first time pure cultures of the tetanus bacillus reduced the etiology of that disease to a clear issue, and when in the next year he, conjointly with Behring, discovered an antitoxin for tetanus in the blood of animals, he helped to lay the foundations of serum therapy, and prepared a way for saving innumerable lives. In 1894 he discovered the bacillus of plague and described with complete accuracy all its essential properties. He advanced his science along many other notable lines. Kitasato first studied Medicine at Tokyo, and in 1885 was sent by the Japanese Government to study under Robert Koch at Berlin, where he was recognised as perhaps the most brilliant disciple of that master. After carrying out in Germany a great number of high-class researches, he returned to Japan in 1891. A few years later the Imperial Japanese Institute for the study of Infectious Diseases was founded by the Government, and was under the charge of Kitasato for fifteen years. It attained to great success and prestige. For his services to science and his country Kitasato was, in 1923, ennobled and given the title of Baron. He was admitted as a Foreign Member of the Royal Society in 1908.

ALFRED BARNARD BASSET, a mathematician, was free from the necessity of adopting a profession and devoted himself to research. From 1897 onwards he produced a succession of papers dealing with such subjects as the equilibrium of revolving fluids, the theory of elastic plates and shells, the variable slow motion of a sphere in a viscous fluid, and with many other aspects of applied mathematics. His work was distinguished throughout by an exceptional command of analytical methods. He was the author of several able treatises which at an earlier period were devoted to applied and later to pure mathematics. He was elected a Fellow of the Royal Society in 1889, and was Vice-President of the Mathematical Society in 1892–93.

Sir OTTO BEIT, though not himself engaged in scientific pursuits, was one to whose enlightened generosity medicine and its allied sciences owe a great debt. In 1909 he established a Foundation as a memorial to his brother, Mr. Alfred Beit, and endowed it with a personal gift of nearly a quarter of a million sterling. This Foundation supports a number of carefully selected research workers, known as Beit Memorial Fellows, whose work during the last thirty years has made valuable contributions to many branches of knowledge bearing directly or indirectly upon the advance of practical Medicine. Few laymen have had such a clear understanding of the necessity and nature of research as Sir Otto Beit possessed, and he took care that the activities of those who benefited by his generosity should be free from inhibitions due to inelastic regulations and requirements. He made other large gifts for the furtherance of clinical medicine itself; in particular, for the provision of adequate supplies of radium at the London Hospitals. His services to science were recognised by the Royal Society in his election to the Fellowship in 1924.

Lord MELCHETT (ALFRED MORITZ MOND) for some years, and especially after the War, was a dominant personality in enterprises which in this country have sought to apply science, and especially chemical science, to industry. He was a youth when his father, Dr. Ludwig Mond, was developing the activities of the great chemical firm of Brunner Mond, so that inheritance and early impressions prepared him for success in the later stages of his career. For a time he practised at the Bar, and in 1906 entered politics, which long occupied most of his time. In 1925, however, he returned to chemical industry, in the development of which he played so great a part. He often expressed his faith that it is the chemist who will solve the present economic and industrial problems of the world. His latest enthusiasm was for the rationalisation of industry, and the great combine, " Imperial Chemical Industries ", was largely an outcome of that enthusiasm.

Sir CHARLES ALGERNON PARSONS. It is due to the genius and firm faith of Charles Parsons that the steam turbine now produces practically all the electricity derived from steam power, and that every fast ship of large size, both naval and mercantile, is driven by steam turbines. This great end was attained slowly and in the face of many difficulties, but the creator of the turbine was never stopped by difficulties, even if at first they might seem insuperable. Apart from his engineering work, Parsons had wide scientific interests. Inherited from his father, Lord Rosse, was a special concern with optical work. He himself devised greatly improved methods for producing search-light reflectors, and the Heaton works, an enterprise started by himself at Newcastle-on-Tyne, came to make nearly all the parabolic reflectors produced in Great Britain. His attempts to make diamonds artificially, though unsuccessful, illustrated his courageous spirit and experimental enterprise. He believed very firmly in the importance of research in industry, and never grudged expenditure upon it. He was the Rumford Medallist in 1902, and in 1928 received the Copley Medal. He was a great man who served his generation nobly.

WILLIAM CARMICHAEL McINTOSH, a marine zoologist, who died at the great age of 92, was for many years engaged in studying the invertebrate fauna of the North Sea. In 1873–74 the Royal Society published the first volume of his great monograph on British marine annelids. This volume dealt with the nemertines, and its great merits received full recognition both in this country and abroad. The remaining volumes of the monograph, dealing with the polychaetes, were unfortunately not published till many years later, and made less impression. In 1882 McIntosh was appointed to the chair of Natural History at St. Andrews and was shortly afterwards selected by a Royal Commission for the duty of enquiring into the problems raised by the introduction of beam trawling and its effect upon the sea fisheries. His keen pursuit of this work led to his establishing at St. Andrews the first marine laboratory in Great Britain. It was a modest establishment, but during its existence it gave opportunities for study to many zoologists of distinction. McIntosh and his pupils played a large part in the researches which showed that all marine food fishes except the herring have pelagic floating eggs. The proof of this, and their careful description of eggs and larval stages, were of importance in their practical bearings. McIntosh received a Royal Medal in 1899.

JAMES LORRAIN SMITH, a pathologist, who is best known to students of science from his early work with J. S. Haldane on hæmoglobin and its relation to carbon monoxide, which led to a practicable method of determining in man the volume of the blood and the total quantity of circulating hæmoglobin. This method was applied by Lorrain Smith himself to the investigation of human anæmias and yielded interesting results by showing that in pernicious anæmia there is a substantial reduction of hæmoglobin, whereas in chlorosis there is no such shortage but only a dilution of the blood with excess of plasma. In conjunction with Mair he devised two histological methods of value—the Nile blue sulphate stain for neutral fats and fatty acids, and an improvement in Weigert's method which made it suitable for the demonstration of tissue lipoids. Smith attained to distinction in philosophy in his undergraduate days and he remained perhaps more a philosopher than an experimentalist. Nevertheless, on the more technical aspects of his subject he did much sound work. He held successively the chairs of pathology at Belfast, Manchester and Edinburgh.

WILLIAM DOBINSON HALLIBURTON. Halliburton was, in this country, a pioneer in the field of physiological chemistry. If he cannot be said to have made fundamental discoveries in that branch of science, he did sound work for it at a time when it was largely neglected alike by English physiologists and chemists. In later years he turned rather to physiological experimentation. His work upon the cerebrospinal fluid, published in conjunction with W. E. Dixon, reached a high order of merit. Halliburton was an admirable teacher and his stimulating influence upon many pupils during his long tenure of the chair of Physiology at King's College, London, was of much service alike to physiology and biochemistry. His "Text Book of Chemical Physiology and Pathology," published so far back as 1901, was then the only work of its kind in English, while other books written by him since, and especially the nineteen successive editions of his "Handbook of Physiology," have long served in the education of medical students. He became a Fellow of the Royal Society in 1891. An unfortunate failure in health removed him from active life for nearly nine years before his death.

CHARLES THOMAS HEYCOCK. Heycock's work upon metals and their alloys, done throughout in conjunction with his lifelong friend, F. H. Neville, and lasting for more than a quarter of a century, was of the utmost scientific importance. The first of the conjoint papers was published in 1889, and dealt with the depression of the freezing points of metals induced by others dissolved therein. Further observations were published in later papers and the authors showed that the addition of small amounts of a second metal depresses the freezing point of the first to an extent which is directly proportionate to the atomic or molecular weight of the added metal. Raoult's law for ordinary solutions was proved to hold for alloys. A method was also indicated for calculating the latent heat of fusion of a metal by an application of the van't Hoff equation to the freezing point depression. The invention by Callendar of the platinum resistance pyrometer rendered valuable assistance to the researches; the melting points of many metals in the pure state were determined, and later those of mixtures of metals in all proportions. The results interpreted by the phase rules of Willard Gibbs and Rooseboom were supplemented by the microscopic examination and photography of the polished and etched surfaces of the solidified alloys. Heycock, like his colleague, was a fine technician, and the work done was of the highest degree of accuracy. It attracted the interest of the Goldsmiths' Company, who endowed a Readership in Metallurgy at Cambridge, an office which Heycock held for twenty years. He was elected to our Fellowship in 1895 and was awarded the Davy Medal in 1920.

HERBERT TOMLINSON, while Lecturer in Natural Philosophy at King's College, London, carried out a series of researches on the influence of stress and strain on the physical properties of matter, on moduli of elasticity for example, and on electric conductivity; and also the effect of magnetisation on the elasticity and internal friction of metals. Between the years 1885 and 1894 he published upwards of forty papers upon such subjects. He was elected a Fellow in 1889.

ARCHIBALD BARR. Professor Barr was an eminent engineer of great inventive ability to whom the country was greatly indebted during the War for the prominent part he took in supplying technical equipment to the fighting services. Many of his own inventions at this time added much to the efficiency of that equipment. One of his first important inventions, worked out in 1888 in conjunction with his then colleague, William Stroud, was the modern range-finder. In 1905 Barr invented a step-by-step motor for signalling electrically from range-finder to gun. In later years other instruments were designed and the intricate fire-control system of the modern warship is based upon Barr's enterprises. In 1889 he returned to his own University of Glasgow as Regius Professor of civil engineering and mechanics. As the results of his efforts the James Watt Engineering Laboratories were opened in the following year. So inspiring was his teaching there that his school at Glasgow rapidly attained to fame.

Walter Ernest Dixon had an international reputation as a pharmacologist, and was a member of the League of Nations Committee on drugs of addiction. His scientific work covers a wide ground involving many studies of drug actions. Outstanding was his work with Brodie upon the bronchial muscles and vasomotor nerves of the lung, directed to explain the nature of asthma; and also his studies of the secretion and circulation of the cerebrospinal fluid. Dixon at one time held the chair of Pharmacology at King's College. He was made Lecturer in his subject at Cambridge in 1909, and ten years later was given the office of Reader.

Sir **Thomas Stanton** as an engineer was noted for his continuous activity in research and for his skill in applying laboratory methods to engineering problems. His personal investigations were made in many fields, including, for instance, the effect of wind pressures upon structures, the resistance of materials to intermittent stresses, lubrication, the heat transfer and friction between solid surfaces and moving fluids, and the movement of projectiles at speeds exceeding the velocity of sound. After holding academic positions at Liverpool and Bristol, he joined in 1901 the Staff of the National Physical Laboratory, where he built up the Engineering Department from small beginnings to its present eminence. For some years he had also charge of the aero-dynamical work of the laboratory.

Percy Groom, a botanist who did valuable work on the morphology of flowering plants, but who will perhaps be best remembered by his application of botanical knowledge to economic problems. He published many papers on timbers and forestry topics, and on the diseases which affect wood in various circumstances. An investigation which was still in progress when he died dealt with mildew as affecting book covers. This was undertaken on behalf of the Public Record Office. He held the chair of the technology of woods and fibres at the Imperial College of Science and Technology.

Sir **Howard Grubb** devoted a long and active life to the design and manufacture of astronomical and other optical instruments. To such enterprises he applied profound scientific knowledge and instinctive ingenuity. His father, Sir Thomas Grubb, who also possessed great skill in the art of lens and mirror making, was elected to the Fellowship of the Royal Society in 1864. Howard Grubb built the 27-inch refractor and three domes for the Vienna Observatory between 1878 and 1881, and during subsequent years produced refractors of large diameters for Cape Town, Oxford, Greenwich, and other observatories. During the early nineties he constructed seven 19-inch photographic refractors for the International Survey of the Heavens, a task which presented exceptional technical difficulties. In connexion with all such enterprises, many and various advances in technique were due to him. In 1905 he invented the submarine periscope which was adopted by the Royal Navy, and he made 94 per cent. of these instruments as used in the War. In later years he was closely associated in both scientific and commercial enterprises with Sir Charles Parsons, upon whose death his own followed so closely.

Sir **David Bruce.** Major-General Sir David Bruce was one of a relatively small band of investigators who, towards the end of the last century, laid solid foundations for tropical medicine. His early work upon the etiology of Malta fever displayed his high qualities as an investigator and led to the practical control of a very troublesome disease. Later he investigated the diseases known as "nagana" and "tsetse-fly disease." These were supposed to be distinct, but Bruce demonstrated their identity, both, as he showed, being caused by a trypanosome. He then proved that the parasite was carried and transmitted by the tsetse-fly. In 1903 he went at the request of the Royal Society to study sleeping sickness in Uganda, and did successful and important work in making clear its etiology.

Before turning to other matters, I cannot refrain from making regretful reference to the illness of our Assistant Secretary, Mr. F. A. Towle. All our Fellows who have had contact with him, or have sought his help in any way, will have recognised the qualities that have made him an admirable permanent official of the Society. He has taken great personal pride in its activities, always being careful, where it lay in his power, to protect its interests and its prestige. To the Officers his organising ability and his grasp of detail have been invaluable. One of his recent tasks, very thoroughly carried out, was the preparation of the new catalogue of the portraits in the possession of the Society. All will most sincerely hope that Mr. Towle's health may be re-established. Mr. R. Winckworth now holds the position of Acting Assistant Secretary.

In reviewing briefly the activities of the Society during the year, I will first refer to the disposal of our Trust Funds. Lord Rutherford, in his Address last year, discussed the reasons for the Council's recent policy in respect of these. A period of conservative treatment of the Funds, justified at the time, had led to an accumulation of capital, and, as stated in its Report of last year, the Council, upon consideration, decided that the time had arrived when the holdings entrusted to the Society should be freely expended, in accordance with the donors' intentions, upon the active support of current researches of fundamental importance. Lord Rutherford described the progress of that policy up to the date of his last Address. This year's Council, as I shall immediately point out, has continued to apply it. That it is justified few, if any, will deny; its results will, I think, prove its wisdom. It should be fully understood, however, that the Society has no longer considerable balances of unallotted income in its hands; not even in its major trusts, such as the Messel and Mond Funds. I am sure, however, that the desire of the Society to do all that is financially possible for the support of individual research nevertheless remains.

The Council has this year made the following grants: From the *Messel Fund:* £800 a year for five years to Dr. Honor B. Fell, of the Strangeways Research Laboratory, for the support of her valuable work on Tissue Culture; also £150 for the current year, and, after the termination of his 1851 Exhibition Scholarship, £600 a year for two years to Dr. M. L. Oliphant, of the Cavendish Laboratory. From the *Caird Fund:* £2,200 to Professor O. W. Richardson for the purchase of optical apparatus of high resolving power. From the *Donation Fund:* £400 to Dr. L. S. B. Leakey towards the cost of his East African Archæological Expedition. From the *Darwin Fund:* £500 a year for four years to Mr. C. S. Elton for research on wild vole populations, together with an additional grant of £250 for capital outlay and field equipment.

I may remark here that Dr. Adler's researches on Kala-azar in Mediterranean countries, mentioned in Council's Report last year, continue to receive support from the Anonymous Bequest Fund.

During the year the Society has received three bequests—one of £10,000 from Sir Otto Beit, a second of £3,000 from the estate of Dr. A. Muirhead, and a third of £275 from that of Dr. C. W. Andrews. These bequests were left without restrictions, and have been added to the General Fund.

It is satisfactory to be able to report that, arrangements with the University of Cambridge being complete, the Cryogenic and Magnetic Research Laboratory referred to in last year's Address will now come into being as the "Royal Society Mond Laboratory." This title secures recognition of the circumstance that the Society was able to forward the enterprise through its possession of Dr. Ludwig Mond's benefaction. Further details of the arrangements will be found in Council's report.

The conditions under which grants have been made to individuals for the purchase of valuable apparatus have hitherto been inadequately defined. Council has been wise, I think, in deciding that the use of such apparatus shall be under the control of a "Scientific Apparatus Committee," in which will be merged the committee which now controls the Society's store of radium. The applicant for whom such valuable apparatus is originally purchased will not be regarded as having a vested interest in it beyond the first full year after delivery.

I do not need to remind you that at the Special General Meeting of Fellows, held at the close of last year, it was decided not to request the Council to alter their decision to modify Statute 5. This statute now so reads as to allow the Council to nominate seventeen candidates annually for election to the Fellowship, and, as you are aware, seventeen new Fellows were duly elected in May last. I believe that those of you who have cared to give further thought to the matter during the months which have since passed, will, whatever your first reaction, have become convinced of the wisdom of this essentially conservative change in our Statutes. There can be no question from what was said at the General Meeting that the Fellows in general desire that the Council should henceforth exercise its statutory power of nomination with greater freedom and over a wider range than has been their custom of late years. More difficult to estimate was opinion concerning the direction of such increase in range. If there be differences of opinion on this point, I would like to state my profound conviction that so great is the sense of responsibility felt and displayed by every member of each successive Council when nominations are the issue, that the interests of the Society itself, like those of every individual candidate, will continue to be safeguarded. In my opinion no real conflict between collective interests and individual claims can ever arise.

I may now be allowed to follow custom and say a few words concerning current scientific progress. My distinguished predecessor, as was natural and proper, dealt in successive Addresses with aspects of physical science. It is for me to turn your attention to biological matters.

Prominent to-day is a call for trained biologists in the economic sphere. They are urgently required for the service of the Empire. A little while ago it was somewhat suddenly realised by administrators that for the successful development of some of our most promising Colonies, and indeed for the solution of not a few economic problems at home, the practical application of a sound biological knowledge had become essential. In many wide districts of the Empire ubiquitous parasites in their legions hold sway over human destiny, and in some districts these diverse legions form the only factor limiting economic progress. The application and the development of biological knowledge can alone solve the problems that thus arise. Tropical climates indeed present many other environmental problems distinct from the example mentioned which call for the aid of biological science. There are to-day indeed still great opportunities for the systematist, the ecologist, the economic entomologist, and for field naturalists in general to apply their knowledge in this service.

It is however of the progress of experimental biology, more closely defined, that I wish in particular to speak. Although at least one generation of investigators has been busy in applying the method of controlled experiment to the analysis of living organisms, yet relatively at least the method is new to biology, and there are still those who for one reason or another mistrust its application. You are doubtless familiar with the philosophic attitude of which we have heard a good deal of late, and which in some quarters has achieved the name of Holism. Most of its implications are doubtless metaphysical, but it is apt to involve explicitly or implicitly a distrust of all attempts to study a living organism otherwise than in its integrity. Apart from those who are obsessed by this holistic philosophy, there are still professed biologists whose minds, being adjusted to morphological and descriptive studies, or to the study of behaviour in the organism as a whole, are out of sympathy with the analytical methods of experiment.

I think, however, it is not going too far to claim that recent progress in experimental biology, though to a superficial view less impressive, has been not less significant, and indeed not less revolutionary than the progress of modern physics. I might support this claim in many ways. It is, I think, justified in that region of knowledge where cytology and genetical studies meet together. It is now, of course, some years since the reaping of significant facts from this field began, but the harvesting actively continues and the store is growing rapidly. That inherited characters as displayed by plant or animal are transmitted as particulate factors, segregated in one generation and regrouped in the next—inheritance being, so to speak, atomic—has become common knowledge since the first rediscovery of the work of Mendel. That each factor or gene so inherited has an objective determinant which can be seen and identified as a material unit of structure in germ or sperm is also, I think, well known. That the progressive behaviour and the interactions of such units of structure can be followed during development; that the presence or absence of certain among them determines sex; that the reason why certain characters are always inherited together as a group, while others may be segregated, is because their material determinants in the egg cell are in the former case visibly associated, while separate in the latter—such facts as these are perhaps familiar to the majority. My purpose in this reference is to emphasise the rapid growth which is to-day proceeding in this domain, and the significance of the facts which are being won on strictly experimental lines. The progress and the significance have become the greater since it was recognised that the material units I have mentioned—the chromosomes and sub-divisions of chromosomes—are "determinants" rather than "carriers" of genetic factors. It is from complex interactions of these factors with the sum total of their environment the final issues arise; but this concession to Holism detracts nothing from the significance of the experimental results. Their significance is perhaps best illustrated in this, that from the chromosome map as seen in the germ cell the display of inherited characters in the final organism may be predicted.

Unfortunately, time will not allow me to illustrate by details the progress I have claimed. I myself, indeed, have enjoyed no personal contact with the converging lines of research which have led to the present fascinating outlook in this region of science. I know enough of them, however, to wish that the facts which they have won and the methods they have used might be widely known among those whose own researches are not concerned with biological problems. They illustrate so well for other experimentalists what is peculiar in the aims, needs and methods and, I would add, the triumphs of experimental biology. One recalls the brilliant Croonian Lecture delivered by Professor T. H. Morgan in 1922 entitled "The Mechanism of Heredity." Here fundamental facts will be found and predictions of that future progress upon the occurrence of which I have insisted.

This domain of disinterested science is making many practical contacts. To mention but a single recent instance: Professor R. C. Punnett, one of the original discoverers of the phenomenon of sex-linkage in inheritance, by applying his expert knowledge of that phenomenon, has produced what may be called a synthetic fowl, of which the qualities are such as to make it of extreme value to the now highly important industry of poultry breeding and egg production. The bearing of the same body of knowledge upon human affairs has been recently very ably discussed by Professor L. T. Hogben.

The phenomena of heredity were long the stronghold of those who cling to the obscurantism of vitalistic doctrines. Infinitely complex as of course they are, we have now abundant proof that they are susceptible to analysis, and that to-day they are yielding their secrets to well-controlled experimental studies. The results of these are becoming quantitative and are even yielding material for mathematical treatment, as the interesting writings of Dr. R. A. Fisher and Professor J. B. S. Haldane have shown.

Another region in which accurate experimentation has removed, and is continuing to remove, inhibitions due to obscurantist assumptions is the physiology of the nervous system of vertebrates. It is, perhaps, too late in the day to refer to the work of Pavlov upon conditioned reflexes, though it is justifiable to emphasise its still growing influence upon thought. The work is a supreme proof of the success of the experimental method in analysing even such apparently transcendental phenomena as those which underlie the higher functions of the brain. This it has done without need of reference to psychical reactions, concerning which, as Sir Charles Sherrington remarked in one of his Addresses from the Chair, it affirms nothing and denies nothing. The nature of the transmission of events in the nervous system is receiving much illumination from the work of each of our Foulerton Professors. Professor Adrian, having developed a most admirable experimental technique for the purpose, is studying the nerve impulse and its origin with highly profitable results. He and his colleagues are now able, with great gain, to work with single nerve fibres and single isolated end organs. The apparatus for recording the transmitted potential changes in these has attained to great perfection. It involves valve amplification, and, when desirable, the conversion of the electric discharges into sound waves by a second power amplifier and a loud speaker. High frequency potential changes are recorded by means of the strikingly efficient oscillagraph designed by Professor Adrian's colleague, Mr. Bryan Matthews.

A striking circumstance, brought to light by Adrian's work and that of his colleagues, is that the nervous structures so far examined exhibit such physical regularity in their behaviour that results can often be predicted within about

1 per cent. His experiments on animals have shown how the phenomenon of grading in the contraction of muscles is controlled by the frequency of impulses sent out from the central nervous system, and by the number of nerve and muscle fibres involved. Moreover, he has been able to observe the activity of a single nerve cell in his own spinal cord by needle electrodes placed in his muscles, and finds that human voluntary contractions are regulated in exactly the same way.

Further, Adrian has found that slow potential changes occur in nerve cells and that these are connected with their discharge of impulses; so far this work only extends to isolated nerve ganglia from insects and to nerve cells in the brain stems of fish, but the phenomena he has found are extremely significant, and it seems possible that changes of potential may be of fundamental importance in the activity of nerve cells.

Adrian has found that damaged nerve fibres set up impulses at very high frequencies and these perhaps play an important part in sensations of pain, though his more recent work has made it clear that impulses in the smaller slowly conducting nerve fibres must also be concerned in the physical mechanism responsible for pain. A most striking feature of all this work is the general similarity of behaviour of nervous structures, from whatever animal they may be taken. A ganglion cell in a caterpillar (I quote Dr. Matthews) seems to work on the same general principles as a nerve cell in Adrian's spinal cord. Nature seems to have hit on sound principles for nervous structures very early in evolution, and to have gone forward to more and more complex arrangements helped by the adequacy and efficiency of these principles.

Our Senior Foulerton Professor is studying the nerve impulse from the standpoint of the thermal phenomena which accompany it. So small, of course, is the heat production in the nerve that its measurement, like that of the potential changes, calls for great refinements. Professor A. V. Hill has conquered the difficulties involved and is revealing to us the magnitude and time relations of the heat discharges involved. It is becoming clear, though we do not yet know the details of its nature, that the nerve impulse consists of a transmitted physico-chemical event, probably involving changes of ionic concentrations at membranes with consequential changes of electric potential; the whole cycle of events, comprising activity and recovery in the nerve, being supported by the energy derived from metabolic oxidative processes which, very small in scale, are associated with the cycle.

When we hope for an increase in our knowledge of the nervous system, we are always accustomed to look to researches from the laboratory of Sir Charles

Sherrington. Perusal of our B Proceedings during the last year will show that such help is still forthcoming in abundant measure. To the results published in the most recent papers from Oxford, those for instance by Professor Sherrington and J. C. Eccles, I may make the briefest reference. An extended study of reflexes has shown that the centripetal impulses do not pass straight through the spinal cord, but at central stations in the cord they are transformed into an enduring excitatory state which may in turn set up fresh nerve impulses yielding the reflex discharges. The nature of this central excitatory state is being studied by ingenious experimentation. The important results will link up, I think, with some of Adrian's observations.

I cannot refrain from mentioning here a recent research which forms a link between my discussion of the nerve impulse and the chemical considerations that I want next to put before you. It has long been suspected, though not proved, that when a sympathetic nerve is stimulated adrenaline is liberated at the nerve ending, and that the observed effects are immediately due to the action of that substance. Now our Secretary, Dr. Dale, in conjunction with a member of his staff, Dr. Gaddum, has investigated the case of the parasympathetic nerves, the influence of which in general opposes that of the sympathetic group, and had obtained good evidence that when one of these is stimulated the substance acetyl-choline, previously existing in some inactive form, is liberated at the nerve ending. The action of acetyl-choline, when injected into the circulation, resembles in general the effect of stimulating parasympathetic nerves, and there is every reason to believe that the physiological activity of the substance, rather than the transmitted physical impulse itself, is immediately responsible for the observed effects of stimulation. I may say that Otto Loewi, of Graz, has shown that when the heart beat is inhibited by vagus stimulation, a substance resembling acetyl-choline in all its known properties is actually formed in the organ, and acetyl-choline, when artificially injected, is known to produce effects like that of the vagus.

In rather unexpected circumstances we have thus brought before us an example of specific physiological effects due to the influence of the specific structure of an organic molecule. Such effects and such relations are being demonstrated in increasing diversity as fundamental factors of organisation in the animal body. This is illustrated most strikingly, of course, in the domain of the control of its activities by a group of hormones. We find in the cases of adrenaline and thyroxin, the constitution of each of which is accurately known, widely different influences depending on differences of molecular structure. The number of known hormones is now increasing and with regard to those which control the sexual and reproductive cycles, illuminating knowledge is increasing fast. I will only mention the hormonic system which correlates the activities of the pituitary, the ovum, and the uterus. In referring to this remarkable case of organisation by means of circulating chemical agencies, it is a pleasure to note how important have been the contributions to the subject which have come from Dr. A. S. Parkes, who is at present working as our Foulerton Research Student.

I may logically pass from a consideration of hormones to devote a few words to vitamins. Although, unlike the hormones, these are of exogenous origin, their activities in the body are equally potent and their functions in general as indispensable. The number of known vitamins has grown to seven or eight and the study of their functions has become a complex matter. At the same time, nobody who is in contact with the evidence doubts the reality and indispensability of these several functions. It is highly satisfactory to know that we are now within measurable distance of knowing the chemical structure of two of these substances.

We now possess proof that vitamin A is closely related to the carotenes and this knowledge may well lead, without long delay, to the artificial synthesis of the vitamin itself. With respect to vitamin D it seems probable, if not yet quite certain, that its artificial production is already accomplished. Some four years ago the constituent of animal and vegetable substances, which is converted into the antirachitic vitamin D by ultra-violet radiation, was identified as ergosterol by Rosenheim and Webster at the National Institute for Medical Research, and concurrently by Windaus in Göttingen. A team of workers at the National Institute, led by Dr. R. B. Bourdillon, appear now to have arrived at the next stage, of isolating the vitamin itself, in crystalline form, from the mixed products of irradiation ; and Professor Windaus, following with his co-workers a different route, has again arrived simultaneously at the same goal. There is no doubt that the substance which the British group now term " calciferol," and which they have isolated as a dinitrobenzoate from the mixed product, is identical with the " Vitamin D_2 " which Windaus and Linsert have obtained by a different method ; and there is little doubt that this substance, as obtained in either laboratory, is the essential vitamin D in a state of practical purity. It appears to be an isomer of ergosterol. One milligramme of calciferol has an antirachitic activity corresponding to about 40,000 of the newly accepted international units.

Such progress in the chemistry of vitamins cannot but be highly satisfactory to all those (and their name is now legion) who have been engaged upon the study of their nutritional functions. Our ignorance of their chemistry and the claim that they function in such infinitesimal amounts left, at any rate till recently, in the minds of many who have not been in touch with the evidence some scepticism as to their real importance. Any disbelief in the significance of this field of investigation is, however, fated to disappear, and I venture to predict that in quite other fields discriminative studies of animal nutrition are yet to yield unexpected results of much practical importance.

Before I close I would like to refer for a moment to a certain aspect of the chemical dynamics of living cells, concerning which progressive studies have been made during the last few years. The cell is, of course, a seat of catalysed chemical reactions and would seem to possess a multitude of catalysts each highly specific in the influence it exerts. In the case of reductions and oxidations, however, though these events, like others, are specifically controlled, there are other agencies of less specific activity which promote the final stages of oxidation. Of great interest among these are certain combinations of metallic iron with pyrrol groupings. One such compound is concerned, possibly with the intervention of yet another, in bringing molecular oxygen into the field of activity. What, however, is specially interesting about these associations of pyrrol groupings with a metal is the wide extent of their biological functions. We have long known, of course, of the presence of one such association in the chlorophyll molecule, where it functions as part of the trap for solar energy, and we have been long familiar with the presence of another in hæmoglobin, where its function is to hold oxygen during its transference from the lungs of vertebrates to their tissues. Further, we now know that, within the tissues, two others promote oxidation, and yet a third prevents by its presence any deleterious accumulations of hydrogen peroxide. For adjustment to each separate function there is some slight modification of a fundamental structure. Compounds of the type in question are found in many of the lower organisms, and I am here led to refer to what was said earlier when nerve activity was under discussion. Just as Nature seems to have hit upon sound principles for nerve structure early in evolution, so she seems to have satisfactorily chosen, very early, the chemical materials for life. This same suggestion is carried by all the more important constituents of living stuff; fundamentally the same throughout, yet always with minor differences underlying specific morphological differences. Each of these aspects, the likeness and the unlikeness, has its own interest.

To return to cell dynamics. Knowledge of enzymic catalysts which, with highly specific relations, activate in a certain sense the molecules which are to suffer oxidation, is almost daily accumulating. It is because this specific activation must precede oxidation that the indiscriminate action of oxygen on the living cell is prevented. Although an understanding of the complex organisation of chemical events in the living unit is far beyond our present powers we are, I think, beginning to see what kind of organisation it may be.

One last word, however. We have assumed that the living cells, the units we have best known, are the ultimate units in biology. But of late years the Viruses have forced themselves into our thoughts. Potent agencies, responsible for fell diseases ; small enough to be outside the range of ordinary microscopic vision, yet in some limited sense at least, capable of self-reproduction. What are Viruses ? Do they merely simulate some of the properties of the living ? Can we conceive of them as something between the non-living and the living ? Are they alive ? We do not yet know. Research upon them is at any rate intensely active at the moment. Its results may make it necessary to modify some fundamental biological concepts, and indeed be as revolutionary in its effects as the breaking up of the atom.

Statement of Award of Medals, 1931.

The Copley Medal is awarded to Sir Arthur Schuster.

Sir Arthur Schuster was the first to show the important information to be got by measuring quantitatively the magnetic deflection of cathode rays. He showed how, by combining this measurement with the potential difference which generates the rays, it was theoretically possible to determine without ambiguity the velocity, and the ratio of charge to mass, of the particles constituting the corpuscular stream. The theory of the method, as he laid it down, is exactly that which has stood the test of time. He was less successful in his own experiments in the application of it mainly owing to having attached too much importance to the possible action of residual gas in depriving the particles of their full velocity. Nevertheless the steps he took were a fundamental contribution to the line of work which afterwards led to the discovery of the electron.

We owe to him other almost equally fundamental contributions to the study of electric discharge in gases. Thus, he showed that the passage of a luminous discharge put the gas temporarily into a conducting state, due to the presence of charged ions : these ions were able to diffuse into a space screened from the discharge by a wire gauze partition, and they could then be put into evidence by showing the conductivity of the gas under electromotive forces of a fraction of a volt. The importance of work like this at a time when ionising radiations were still unknown needs no emphasis.

Schuster was the first to show by experiment that in Crookes' radiometer the reaction was not on the sun but on the glass case of the instrument, thereby connecting the action with the residual gas.

He has made many important contributions to terrestrial magnetism. In spectroscopy he formulated independently the Rydberg-Schuster law. He invented the periodogram method of looking for periodicities in statistical material. "The periodogram," he says, "supplies by calculation the transformation which the spectroscope instrumentally impresses on a luminous disturbance." The method has been widely adopted by workers in many branches of enquiry, extending even into economics.

Sir Arthur Schuster's long and valuable services to the Society can never be forgotten.

A Royal Medal is awarded to Sir Richard Glazebrook.

For fifty years Sir Richard Glazebrook has been closely identified with research on physical standards, and particularly electrical standards. For many years he conducted researches associated with the absolute measurement of resistance, current and inductance, and the results of this work, together with his continued interest, is reflected in the present remarkable accuracy of electrical measurements. The name of Sir Richard Glazebrook is also world-known on account of his Directorship of the National Physical Laboratory, in the organisation and development of which he was from the first the active leader ; it is largely due to his influence on the researches at that Institution that aeronautical science has made such vast progress. Physical science is also indebted to him for that great work, the "Dictionary of Physics," and in international science he has played a conspicuous part.

A Royal Medal is awarded to Professor William Henry Lang.

Dr. Lang's work on the fossils of the Old Red Sandstone is of high scientific importance. It has led to the discovery and description of a new and unexpected group of plants in which root, stem, and leaf are not differentiated. For the first time it thus becomes possible to trace in a circumscribed group the probable origin of these structures from a source in which they did not exist as distinct members.

The work was begun in collaboration with the late Dr. Kidston, and continued by Professor Lang after the death of his colleague in 1924. Professor Lang's previous intensive studies on the morphology of the Liverworts and

Ferns—in itself a valuable contribution to our knowledge of plant morphology in general, and to the supply of a clearer conception of the interrelationships of the main groups—had eminently fitted him to provide a morphological viewpoint which has given most important results.

The Davy Medal is awarded to Professor Arthur Lapworth.

Professor Arthur Lapworth's work has been largely concerned with the application of physical methods to the investigation of the reactions of organic chemistry. His researches are distinguished by the simplicity and elegance of the experimental methods which he has employed, and by the insight which he has shown in elucidating the mechanism of chemical change in carbon compounds. His investigations have contributed notably to the deeper understanding of the processes concerned in the chemical reactions of organic compounds.

His study of the bromination of acetone yielded results of primary importance in relation to the reactivity of carbonyl compounds and has formed the basis of many subsequent investigations.

His researches on the addition of hydrocyanic acid to organic compounds, besides leading to results of theoretical and synthetical importance, made clear the mechanism of the formation of cyanhydrins.

His investigations of the effect of small quantities of water in diminishing the activity of acids in alcoholic solution indicated the existence of the oxonium ion, and added considerably to our knowledge of catalysis by acids.

Among his more notable synthetical achievements are the synthesis of zingerone, derived from the pungent principle of ginger, and of homocamphor.

His work on the mutual influence of groups in the same molecule, his recognition of induced alternate polarity, and his classification of reagents as anionoid or kationoid have played an important part in the development of the present state of knowledge of the reactivity of organic compounds.

The Sylvester Medal is awarded to Professor Edmund Taylor Whittaker.

Professor E. T. Whittaker is one of the best known of British mathematicians, his work showing extraordinary versatility. He has written five books, on entirely different subjects, and some 50–60 papers which touch on almost every branch of mathematics. His book " Modern Analysis " (now Whittaker and Watson) is a standard treatise in its 5th edition; his " Analytical Dynamics " and his tract on " Optical Instruments " have both been translated into German; and he has also written a " History of the Theories of Aether and Electricity," and a " Calculus of Observations." All these works show, besides their more technical qualities, powers of arrangement and exposition of a most unusual order; and the "Modern Analysis" in this country, and the "Analytical Dynamics" both in this country and abroad, have had a considerable influence on mathematical thought.

It is only possible to mention a few of Whittaker's original contributions to knowledge (beyond the considerable number absorbed in his books). He has made important additions to the theory of the solution of differential equations, ordinary and partial, by definite integrals; to the theory of Lamé and Mathieu functions, the functions of the elliptic and parabolic cylinders, and the integral equations associated with them; to the theory of interpolation; and to the theory of the solution of dynamical problems by trigonometrical series. He has also in recent years made a number of interesting contributions to the pure mathematics of relativity, electromagnetism and quantum theory.

The Hughes Medal is awarded to Professor William Lawrence Bragg.

Professor W. L. Bragg's recognition of the fact that the Laue diffraction spectra could be considered as produced by reflection from the planes of the crystal lattice, besides being a great simplification of a difficult geometrical problem, was the starting-point of two important and fruitful lines of physical investigation, namely, the measurement of X-ray wave-lengths and the elucidation of crystal structure. Work on the first of these led to Moseley's discoveries and their subsequent developments. Bragg's concentration on the second has resulted in a wonderful extension of our knowledge of the structure of crystals, both simple and complex, and of inter-atomic distances and linkages. His work may truly be said to have laid the foundations of a chemistry of the solid state.

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Effect of X-ray Sterilisation on Œstrus in the Ferret.

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[Plate 38.]

1. *Introduction.*

Complete obliteration of the Graafian follicles in the mouse by exposure to X-rays does not suppress the œstrous changes in the uterus and vagina (Parkes, 1926–27; Brambell, Fielding and Parkes, 1927–28; Schugt, 1928; Geller, 1930). It is evident, therefore, that in this animal the actual elaboration of œstrin is not dependent upon the presence of the follicular system. Similar results have been reported on the rat (Ford and Drips, 1929). In the guinea-pig, on the contrary, Genther (1931) finds that the uterine and vaginal changes invariably cease after total obliteration of the follicles. Such of her irradiated animals as showed œstrus all possessed at least one large follicle.

In view of the variation in mammalian ovaries, a species difference in the response to X-irradiation might well be expected; in order to investigate this point, experiments were begun two years ago on an animal with a type of ovary and cycle distinct from that of the mouse, *i.e.*, the ferret. The results are described below.

II. *Technique.*

Irradiation.—The intensity of the irradiation was the same as that used for mice (Parkes, 1926–27). The whole body was irradiated. An exposure of 80 minutes, the largest dose used for the mice, was found not to disturb the general condition of adult ferrets, but young animals were much more sensitive. One litter of six females given 80 minutes' exposure at 11 days old all died within 17 days. Another of three females given two 80 minutes' exposures, at 23 and at 29 days old, all died within 14 days after the second dose. In both groups numerous lesions of the viscera were found on post-mortem examination. Because of this mortality, the length of one exposure for immature animals was reduced to 20 minutes. Adult animals were, however, found most suitable, several receiving four 80 minutes' doses in 3 weeks without any noticeable ill effect. Irradiation was carried out during the anœstrous period. Dates are given where relevant to show the relation to the breeding season.

Detection of Œstrus, etc.—The normal reproduction processes of the ferret have been described by Marshall (1904) and by Hammond and Marshall (1930). The animal is notable for having a definitely restricted breeding season, lasting from March to the end of July. Ovulation takes place only after copulation, and œstrus, persistent for some months in the absence of copulation, is characterised by great swelling of the vulva. The enlargement disappears completely during pregnancy, lactation and anœstrus. Tracings of the swelling were made on glass as recommended by Marshall, and vaginal smears were also taken.

Histological Technique.—Bouin's fixative was used as a routine. Complete serial sections (7 μ), stained in hæmatoxylin and eosin, were made of all ovaries.

III. *Physiological Effects of Sterilisation.*

Three ferrets (F.1, F.7, F.8) were irradiated in the first batch. Each received four 80 minutes' exposures at weekly intervals between January 11, 1929, and February 1, 1929, *i.e.*, just before the breeding season was due to start. Within a month all three had come into œstrus. On March 1, 1929, the left ovary of F.7 was removed. This ovary was found not to be sterile. All three animals remained in œstrus till July, except that the vulva of F.7 subsided temporarily after the unilateral ovariectomy. F.1 and F.7 were mated to a normal dog on June 3, 1929, and May 8, 1929, respectively, but neither became pregnant. At the end of the breeding season (July 19, 1929) each animal was again given 80 minutes' exposure. During the following anœstrus (September 20, 1929) F.1 was killed. The ovaries were found to be completely sterile, while the uterus was of the ordinary anœstrous type. Just before the next breeding season the two remaining ferrets of this group were given two more exposures of 80 minutes (January 14, 1930, March 5, 1930). The animals were kept under close observation during what would have been the next breeding season, but only a very slight swelling of the vulva was observed in F.7 and this only late in the season. In F.8 no external change whatever occurred. The animals were killed on June 2, 1930, and July 17, 1930, respectively. The ovaries were large and fleshy, while the uteri were large and reddish-purple in colour. Histological examination of the ovaries showed complete absence of organised follicles and of oocytes in both animals (see Section IV). The uteri in section showed no signs of ovariectomy atrophy ; they were, in fact, fully up to normal size. The endometrium was similar to that found in early œstrus, differing only from the full œstrous condition in that the hypertrophy of the basal glands was not complete. F.7 was the more advanced of the two (Plate 38, fig. 1). There was no sign of the pseudo-pregnant condition.

The next group of animals (F.35, F.36, F.37) was given a first exposure of 80 minutes, at about 10 weeks old on July 5, 1929. This dose was repeated on September 9, 1929, November 8, 1929, January 7, 1930, and February 21, 1930, the aim being to sterilise immature animals before the onset of their first breeding season. No vulval change was observed in any of these animals till early in May, when a slight enlargement took place in F.37. At killing on June 2, 1930, F.37 retained the slight swelling, but F.35 and F.36 still showed no sign. The ovaries of all three were fleshy in appearance, those of F.35 and F.36 being noticeably large. The uteri of these two were small and pink, while that of F.37 was large and purplish. Histological examination showed that the ovaries of all three animals were completely devoid of organised follicles and oocytes. The uteri of F.35 and F.36 were small and undeveloped (Plate 38, fig. 2), and very similar to that of the anœstrous animal. The uterus of F.37, on the other hand, was very well developed, the endometrium approximating closely to the normal œstrous condition. The difference in the condition of the uteri is curious in view of the identical treatment, but it may be correlated with the difference in the ovarian condition described in Section IV. The following conclusions may be drawn from these six ferrets in which organised follicles were completely absent :—

(*a*) Assuming the probability that F.1, F.7 and F.8 were incompletely sterilised during their first post-irradiation breeding season, it is necessary to conclude that ablation of the follicles in the ferret depresses the production of œstrin to a level insufficient to cause more than a trifling development of the vulva.

(*b*) The uterine findings, however, are rather different. In three of the five animals killed during what would have been a breeding season, the uterus was well developed, and in two showed almost complete œstrous changes. From this result, it may be deduced that the threshold value of œstrin is lower for the uterus than for the vulval swelling, a finding in keeping with the results obtained by the injection of œstrin after ovariectomy (Parkes, 1930).

(*c*) The complete absence of even a trifling change in the vulvæ of F.8, F.35 and F.36, together with the partial castration effect observed in the uteri of F.35 and F.36 are probably correlated with the more extensive ovarian damage in these animals.

The remaining five animals were incompletely sterilised. Two (F.28 and F.29) were exposed for 20 minutes on July 12, 1929, at 21 days old. Three subsequent doses of 80 minutes were given on November 1, 1929, January 28, 1930, and February 28, 1930. No change in the vulvæ was observed until May, when F.28 developed about half the normal œstrous swelling and F.29 a very slight enlargement. At the time of killing (June 2, 1930) the vulval symptoms were about the same. The ovaries of both were small; the uterus of F.28 was pink and of F.29 reddish-purple. Histological examination showed one follicle in each ovary of F.29 and a number of follicles in the ovaries of F.28. The uteri of both ferrets were well developed, but the growth of the characteristic basal glands was much further advanced in F.28.

In these two animals, therefore, the development of œstrous symptoms in both the uteri and vaginæ was directly proportional to the incompleteness of the sterilisation.

The last three ferrets were adults given three 80 minutes' doses on October 25, 1929, January 21, 1930 and February 25, 1930. All the vulvæ began to swell during April, and by the beginning of May were fully developed. At autopsy, a month later, the vulvæ were still fully up. Histological examination showed numerous follicles in all the ovaries, while the uteri were of the normal œstrous type. Taken in conjunction with the previous animals, the following conclusions may be drawn from the incompletely sterilised ferrets :—

(*a*) Under the conditions obtaining, not less than five 80 minutes' irradiations at the standard intensity used are required to produce sterility as diagnosed by the complete absence of organised follicles and oocytes. Decreasing dosage results in the survival of an increasing number of follicles.

(*b*) The more follicles surviving in the ovary, the more normal become the œstrous changes in the uterus and vulva.

A summary of the experimental results is given in Table I.

Table I.—Summary of Results of X-irradiation of Ferrets.

No. of ferret.	Age at first irradiation.	No. of doses of 80 mins. each.	Time spread over.	Development of vulva during breeding season. (+ + + = full.)	Time of autopsy after last dose (weeks).
F.8	Adult	7	14 months	− + + first season after four doses. o second season after seven	17
F.7 (autopsy)	,,	7	,,	+ second season after seven doses	20
F.1	,,	5	6 months	+ + + after four doses	10
F.35	9 weeks	5	7½ months	o	14½
F.36	,,	5	,,	o	14½
F.37	,,	5	,,	+	14½
F.7 (left ovary)	Adult	4	3 weeks	+ + + after four doses	7
F.28	3 weeks	3¼	7½ months	+ +	13½
F.29	,,	3¼	,,	+	13½
F.56	Adult	3	13 weeks	+ + +	13½
F.57	,,	3	,,	+ + +	14
F.58	,,	3	,,	+ + +	14

Table I.- (continued).

No. of ferret.	Ovary.					Uterus.		Vulval swelling at autopsy.	Notes.
	Weight (gms.).	Sterile.	Follicles with oocytes.	Hypertrophied interstitial.	Atretic corpora lutea.	Diameter (mm.).	Condition.		
F.8	—	Yes	o	+++	+	2·6	Similar to pro-œstrus	o	—
F.7 (autopsy)	0·18 (right only)	,,	o	−++	o	2·7	Œstrous	+	—
F.1	—	,,	o	o	o	1·4	Anœstrous	—	Killed during anœstrus.
F.35	0·21	,,	o	+++	o	1·3	Similar to anœstrus	o	—
F.36	0·18 (left only)	,,	o	+−++	+	1·5	,,	o	—
F.37	0·15	,,	o	o	o	2·4	Œstrous	+	—
F.7 (left ovary)	—	No	+−+	+−	o	—	—	—	Ovary obtained by operation.
F.28	0·10	,,	−+	−+	+	2·6	Œstrous	++	—
F.29	0·09	,,	+	++++	o	2·5	Similar to pro-œstrus	+	—
F.56	0·06	,,	+++	+	o	2·2	Œstrous	++−	—
F.57	0·07	,,	+++	+	o	2·5	,,	+++	—
F.58	0·05	,,	+++	+	o	2·4	,,	+++	—

IV. *Ovarian Changes in the Ferret Ovary following Irradiation.*

The peritoneal epithelium of all the ovaries examined resembles that of the normal adult ovary of the ferret and does not exhibit any signs of more active proliferation. The tunica albuginea is also similar to that of the normal ovary. Small anovular follicles are present in the peripheral regions of all the ovaries, but are not very numerous. They are invariably small in size and devoid of an antrum. They tend to be rather more numerous in sterile ovaries than in those containing follicles with oocytes.

Follicles containing oocytes are entirely absent from the ovaries of F.1, F.8, F.35, F.36, and from the right ovary of F.7. In a few cases a small amorphous residual mass in the centre of small anovular follicles probably represents the remains of the zona pellucida of the degenerate oocyte; this suggests that the anovular follicles arose from small follicles by the degeneration of the oocyte. The above ovaries may be regarded as completely sterilised by the radiation, according to the most stringent standard of absence of follicles containing oocytes. One ovary of F.37 is also devoid of follicles but the other contains two, possibly more, large and highly abnormal follicles. These follicles almost entirely fill the region within the tunica albuginea, leaving only a small zone of cortical stroma at one side. The walls of the follicles are so much folded as to render it difficult to determine the number of follicles involved or their limits. The membrana granulosa in these follicles has proliferated in a remarkable manner, producing a highly abnormal appearance suggestive of an epithelioma. The cavities of these follicles are filled with a coagulum, staining like that produced from liquor folliculi. No traces of oocytes are discernible in these follicles.

One large follicle containing an oocyte, but showing definite signs of degeneration, is present in each ovary of F.29, and there is also a small cyst present in the hilum of one of them.

Both large and small follicles containing oocytes are present in all the other ovaries (left ovary of F.7, both ovaries of F.28, F.56, F.57, F.58). The vast majority of these follicles exhibit various stages of degeneration either of the oocyte alone or of both the follicle and oocyte. In a few cases it is not possible to be sure whether the follicle and contained oocyte are normal or degenerating. The ovaries of F.56, F.57 and F.58, which are comparable in that they were irradiated at the same age, received the same dose of X-rays, and were killed the same time after irradiation, exhibit a strikingly similar histological appearance in every respect. They all contain numerous follicles, of which several in each ovary are over 1 mm. in diameter, and the majority, if not all, are degenerating. Further, they are similar in the complete absence of corpora lutea and in the appearance of the stroma, as well as being approximately equal in size. No corpora lutea are present, but fibrous remains, containing scattered luteal cells, possibly derived from corpora lutea atretica, are present in one ovary of F.8 and F.36, and in both ovaries of F.28.

The right ovary of F.7 contains a large fibrous mass which occupies the greater part of the ovary and resembles a fibroma.

The ovary of the normal ferret contains numerous blocks and strands of so-called "interstitial" cells in the cortical stroma. The cells, which are larger than the surrounding cells, probably arise from the walls of follicles that have degenerated. They are present in all the X-rayed ovaries and form relatively a much larger part of the ovary than in the normal animal. They constitute by far the greater part of the ovary in those cases where follicles are absent or few and small. The cells composing this tissue exhibit various stages of enlargement and vacuolation probably caused by fatty infiltration. This tissue in F.1 and F.37 is similar to that of the normal ovary, but the cells composing it are slightly enlarged in F.56, F.57 and F.58, and more definitely so in F.28 and the left ovary of F.7. The cells are larger in all the other ovaries, especially so in F.29 and F.36, and are much vacuolated, except in F.29. The tissue in these latter ovaries somewhat resembles luteal tissue and the condition is evidently similar to that described in mice irradiated at birth.

Size of the Sterilised Ovaries.—The ovaries of the normal œstrous ferret weigh about 0·150 gm. Of the present series the weight of F.7 right ovary may be partly due to compensatory hypertrophy after the left ovariectomy. The remaining seven pairs of ovaries, for which weights are available, all weigh less than normal, except F.35 and F.36. It is curious that two out of the three weighed pairs of sterilised ovaries should be larger than normal, whereas the partially sterilised ones are smaller than normal. The ovaries of F.1 (not weighed) were very small, suggesting that anœstrous hypotrophy takes place in the sterilised ovary.

V. *Discussion.*

In the mouse, the uterine changes of œstrus require a much greater concentration of œstrin than do the vaginal changes (Marrian and Parkes, 1930). Since the uterine as well as the vaginal cycle persists after sterilisation in this animal, the œstrin production by the sterilised mouse ovary must be up to the normal level. In Genther's guinea-pigs, on the other hand, the absence of even vaginal cornification suggests that the completely sterilised guinea-pig

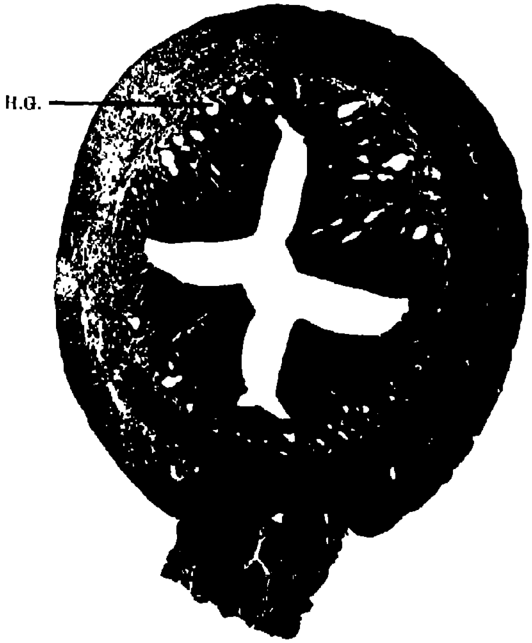

FIG. 1

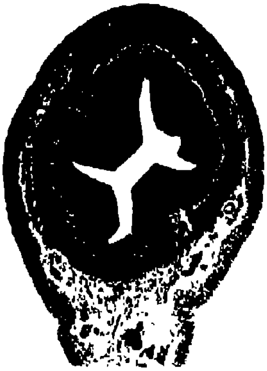

FIG. 2.

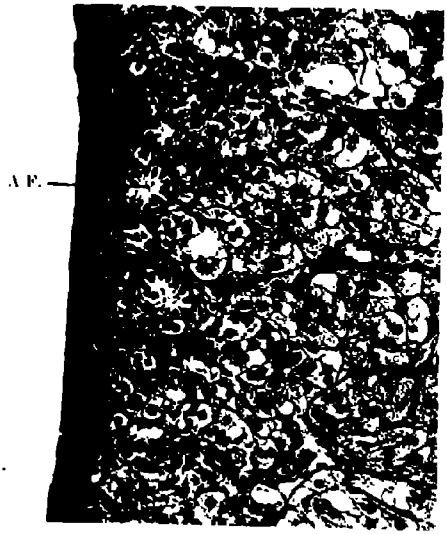

FIG. 4.

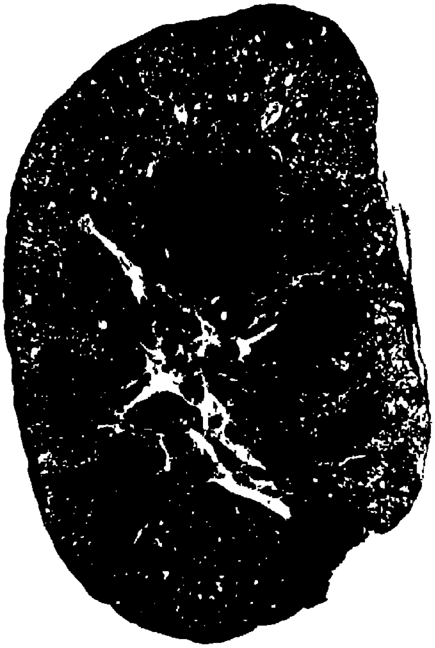

FIG. 3.

ovary fails to produce any appreciable amount of œstrin. From the results described above, it is evident that the sterilised ferret can produce enough œstrin to cause the typical uterine changes, but not enough to cause more than a trifling degree of vulval enlargement.

It is significant that the uteri showed no trace of pseudo-pregnant development in spite of the presence of very large amounts of interstitial tissue in the sterilised ovaries. The ferret, therefore, seems to be intermediate between the mouse and the guinea-pig as regards the effect of obliteration of the follicles on the endocrine function of the ovaries.

It should be emphasised that the different results obtained on different species are not antagonistic; all depends on the exact response of the ovary to the particular dose and type of irradiation used.

We are greatly indebted to Professor G. Elliot Smith, F.R.S., in whose department the irradiations were carried out.

The expenses were defrayed from a grant from the Medical Research Council to A.S.P.

VI. *Summary.*

(1) Complete obliteration of the follicular system of the ferret ovary is produced by five 80 minutes' exposures to X-rays of the type and intensity previously used on mice (Parkes, 1926–27).

(2) Five animals were completely sterilised during anœstrus. Œstrous enlargement of the vulva during the next breeding season was trifling in two and completely absent in three. The œstrous development of the uterus, however, was complete or nearly complete in three of the five.

(3) It may be said, therefore, that the sterilised ovary of the ferret produces enough œstrin to cause the uterine changes, but not enough for the vulval swelling.

(4) A species difference has already been shown in the effect of X-ray sterilisation on the endocrine activity of the ovary (Genther, 1931), and the present results on the ferret in no way conflict with our previous work on the mouse.

DESCRIPTION OF PLATE.

H.G., hypertrophied basal glands of the uterine endometrium. *A.F.*, small anovular follicles.

PLATE 38.

FIG. 1.—Uterus of F.7, showing close approximation to the œstrous condition. × 30.

FIG. 2.—Uterus of F.35, showing undeveloped condition (compare anœstrous uterus, Plate 15, fig. 3, Parkes, 1930). × 30.

FIG. 3.—Whole ovary of F.36, showing general nature of sterilised ovary. × 15.

FIG. 4.—Periphery of ovary of F.36, showing absence of oocytes, and presence of small anovular follicles. × 180.

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541 . 135 . 5 + 576 . 341

The Electrical Properties of the Inner Epidermis of the Onion Scale.

By R. J. Pumphrey, B.A., The Zoological Laboratory, Cambridge.

(Communicated by J. Gray, F.R.S.—Received October 24, 1931.)

Michaelis (1927–28), in his description of the properties of the dried collodion membrane, concluded that it behaved like a sieve permeable in different degree to the hydrogen ion and ions of the Li-Cs series and impermeable to anions. He records, however, that the ratio of the mobilities of univalent cations and anions which is very high in dilute solution decreases as the concentration is increased, a fact which does not fit in with the simple sieve theory. In another paper (Weech and Michaelis, 1929) he found from potential measurements that, when the collodion membrane separated N/10 and N/100 $CaCl_2$, the transport numbers of $Ca^{\cdot\cdot}$ and Cl' were approximately the same as in free solution and concluded that the low value of the mobility of the $Ca^{\cdot\cdot}$ ion was probably due to its large size, *i.e.*, that the obstruction to its passage was purely mechanical. He states, however, that the measurements with calcium chloride were not entirely satisfactory.

In another place (Pumphrey, 1931) the writer has described experiments which suggested that a rather different interpretation of these facts was possible and that a further examination of the effect of concentration on the permeability of membranes of this type was desirable, particularly for salts containing a bivalent cation. In this paper experiments with the inner epidermis of the onion scale are described. As will be shown, this membrane appears to resemble the collodion membrane in its electrical properties and is very much easier to prepare and mount.

A paper on the properties of the onion scale epidermis has already been published (Brooks, Giese and Giese, 1931). These authors find a very variable potential across the membrane which falls off in an irregular manner with time. The present writer has not been able to confirm their results. If he has correctly understood the description of their method, the solutions in contact with the membrane were connected with the calomels by means of capillary tubes with straight ends filled with saturated KCl. It is easy to show that as soon as a capillary containing saturated KCl is dipped into a more dilute solution the KCl solution falls to the bottom and is replaced by the dilute solution, even if the capillary is very fine. The slow fall of potential described by these authors can easily be accounted for by contamination of the solution in contact with the membrane by strong KCl, and the minor irregularities may perhaps be due to fluctuating diffusion potentials in the capillary.

Material.

The inner epidermis of the onion scale is a single layer of cells with thickened cutinised walls easily stripped off intact from the scale and free from stomata. Occasionally it happens that some of the cells are left behind in stripping and only the external cell-wall comes away. This makes no difference to the behaviour of the membrane except that it is more fragile and less easy to handle. It is evidently on the inanimate cell-wall that the properties to be described depend.

Method.

The membrane, after washing in distilled water, was mounted between two tubes with upturned ends and ground flanges, which were held together by paper clips, fig. 1.

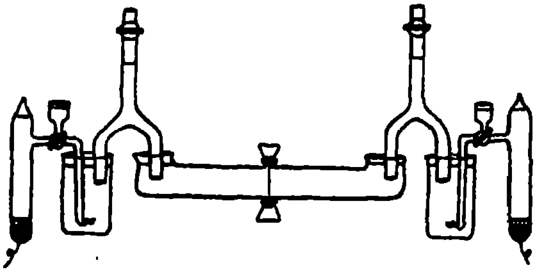

FIG. 1.

In the first experiments the flanges were previously coated with vaseline, but this was subsequently found to be unnecessary. After further washing with distilled water the two halves of the cell were rinsed and filled with the solutions to be used and each half connected by a syphon to a small beaker containing the same solution into which a saturated calomel electrode was dipped. One electrode was connected to the needle of a Lindemann electrometer, the other to one terminal of a slide-wire potentiometer of which the other terminal was earthed. The sensitivity of the system was about 0·5 millivolts. Readings are given to the nearest millivolt. The use of the electrometer has the advantages that no current is taken from the system and that the sensitivity is independent of the resistance of the circuit with the concentrations used.

RESULTS.

I. *Comparison with the Collodion Membrane.*

It was found that different membranes even when taken from the same scale of the same onion gave somewhat different results. In fig. 2 the potential given by different membranes separating solutions of potassium chloride, with a concentration ratio of 1 : 10, are plotted against time. The form of the curve is always much the same. After a rise of variable extent and duration a constant potential is reached which remains constant as long as measurements are continued (in some cases over 18 hours).

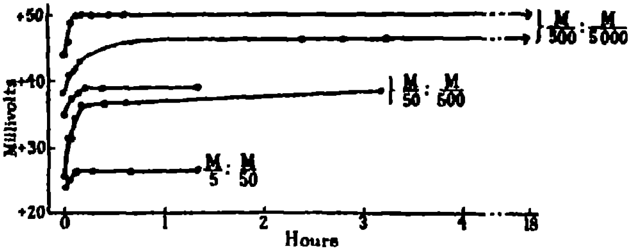

FIG. 2.

(In this figure and subsequently the sign of the potential is that of the more dilute solution.)

Zero time on the graph is the time of the first reading, usually about 3 minutes after the membrane had been in contact with the solutions. From these curves it can be seen that the onion skin in dilute solution may give a potential fairly close to the maximum (58 millivolts), the dilute solution being positive. It therefore resembles the collodion membrane in being nearly impermeable for anions in dilute solution. It was found subsequently that the initial low potentials were seldom observed if the membrane after mounting was washed for several hours with changes of distilled water. The steady potential was then reached almost instantaneously. The initial low values are attributed to traces of electrolyte in the membrane probably derived from injured cells.

Trial showed that any membrane always gave the same potential within 2 mv. with the same solutions of KCl even after exposure to strong solutions of KCl, HCl or several other salts, provided it was first thoroughly washed. It can, therefore, be assumed that in the following experiments the effects of increased concentration are reversible.

II. *The Change of Potential with Concentration.*

(*a*) *Potassium Chloride.*—In this and subsequent experiments a concentration gradient of 5 : 1 was used. Solutions more dilute than 0·0032 M : 0·00064 M gave rather irregular results, probably owing to contamination by traces of salts leaking from the cells of the epidermis. However, at this concentration (*i.e.*, 0·0032 : 0·00064 M) many of the membranes gave potentials approximating to the theoretical maximum (38 mv. at 20° C.).

As the concentration is raised the potential falls nearly to zero, Table I.

Table I.

Concentration KCl.		Membrane 4.	Membrane *b*.	Membrane *c*.
M	M			
0·0032	: 0·00064	28	34	35
0·016	: 0·0032	28	31	32
0·08	: 0·016	24	27	26
0·4	: 0·08	14	16	14
2·0	: 0·4	2	2	1

(*b*) *Hydrochloric Acid.*—Similar experiments with HCl gave the results in Table II. Owing to the possibility of liquid junction potentials of appreciable magnitude existing between the calomels and the acid solutions, it was considered advisable to measure the liquid junction potential between the acid solutions concurrently with the measurements of the potentials across the membrane. This was done by inserting a syphon connecting two beakers containing the acid solutions in place of the membrane cell, fig. 3. This rather simple method gave very constant potentials slightly lower than the values calculated.

Table II.

Concentration HCl.		Membrane 30.	Membrane 38.	l.j.p.
M	M			
0·0032	: 0·00064	37	33	24
0·016	: 0·0032	30	26	25
0·08	: 0·016	23	22	24
0·4	: 0·08	22	22	24
2·0	: 0·4	20	23	22

One of these membranes was a "good" membrane. The other gave a potential considerably lower than the theoretical for the most dilute solution. In both cases the potential across the membrane and the liquid junction potential coincide within the limits of error for the stronger solutions.

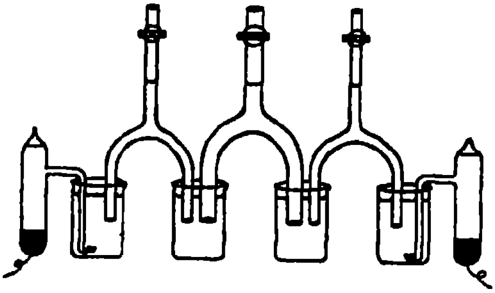

Fig. 3.

(c) *Calcium Chloride.*—There is no satisfactory theoretical equation for the calculation of liquid junction potentials involving a bivalent and a univalent ion. In Table III are given the potentials for two membranes and the mean of two determinations of the l.j.p. for $CaCl_2$.

Table III.

Concentration $CaCl_2$.		Membrane 29.	Membrane 30.	l.j.p.
M	M			
		mv.	mv.	mv.
0·0032	: 0·00064	35	38	(19)
0·016	: 0·0032	21	28	−14·5
0·08	: 0·016	2	8	−14·5
0·4	: 0·08	−12	−11	−12·5
2·0	: 0·4	−15	−16	− 9·5

The mean value for the l.j.p. agrees very well with Michaelis' finding of −18 mv. for N/10 : N/100 $CaCl_2$ (18 log 5 = 12·6).

From a consideration of Table III it can be seen that in dilute calcium chloride the membrane behaves as if impermeable for anions; at a concentration 0·4 : 0·08 it does not significantly alter the normal ratio of the mobilities and is presumably equally permeable for the cation and the anion, and at the highest concentration it appears to be more permeable for anions than for cations. If this membrane is really of the same type as the collodion membrane, it seems likely that the agreement which Michaelis found between the potential across the membrane and the l.j.p. for $CaCl_2$ was fortuitous and would not have been observed at any other concentration. This implies that the size of the $Ca^{\cdot\cdot}$ ion is not the factor that determines its mobility in the membrane.

(*d*) *Cerium chloride* gives very much the same type of results (Table IV) as calcium chloride, but as the salt is hydrolysed to a considerable extent, the interpretation is difficult.

Table IV.

Concentration $CaCl_2$		Membrane 32.	Membrane 33.	l.j.p.
M	M			
0·0016	: 0·00032	34	28	−16
0·008	: 0·0016	32	28	−16
0·04	: 0·008	20	1	−17
0·2	: 0·04	− 8	−17	−17
1·0	: 0·2	−25	−26	−17

It has been shown that when the onion epidermis separates two different solutions of potassium chloride, it gives a constant and reproducible potential. If the membrane acted purely as a sieve, addition of an equal amount of a salt containing a bi- or trivalent cation to each of the solutions in contact with the membrane should not affect the potential except in so far as it (*a*) altered the total concentration of ions to which the membrane was permeable, (*b*) altered the activity of the potassium ions already present.

In the following experiments membranes which reached a constant potential when separating M/50 and M/250 KCl were exposed to similar solutions which were M/1000 with respect to $CaCl_2$ and $CeCl_3$. The results are shown in Tables V and VI respectively.

Table V.

Concentration.	Membrane 59.	Membrane 60.	Membrane 61.
KCl M/50 : KCl M/250	33	34	30
$\left.\begin{matrix}\text{KCl M/50}\\ \text{CaCl}_2\text{ M/1000}\end{matrix}\right\}:\left\{\begin{matrix}\text{KCl M/250}\\ \text{CaCl}_2\text{ M/1000}\end{matrix}\right.$	18	16	18

Table VI.

Concentration.	Membrane 62.	Membrane 63.	Membrane 64.
KCl M/50 : KCl M/250	27	34	29
$\left.\begin{matrix}\text{KCl M/50}\\ \text{CeCl}_3\text{ M/1000}\end{matrix}\right\}:\left\{\begin{matrix}\text{KCl M/250}\\ \text{CeCl}_3\text{ M/1000}\end{matrix}\right.$	6	12	8

It can be seen that the depression is too large to be accounted for if the membrane is a simple sieve. If the membrane remained nearly impermeable to anions the depression to be expected is only about 4 mv. even if $Ca^{\cdot\cdot}$ and $Ce^{\cdot\cdot\cdot}$ ions penetrate as readily as K ions. The activity correction probably amounts to less than this. Actually the depression is 15 mv. for calcium and 21 mv. for cerium chloride.

Discussion.

The results above agree very fairly with Michaelis' results with the collodion membrane so far as univalent salts are concerned. When the potential for a given concentration gradient is plotted against the logarithm of the concentration the curve is sigmoid, the upper asymptote being the potential given by $E = RT/F \ln c_1/c_2$ and the lower by the l.j.p. in the absence of the membrane. With salts containing a bi- or trivalent cation the results are different, the upper asymptote is the same, but for high concentrations the dilute solution becomes more negative than it would be in the absence of the membrane. Fig. 4, which illustrates this point, is based on the figures given above, but somewhat idealised for the sake of clarity.

It is reasonable to regard this fall in the potential as due to the increasing adsorption of cations as the concentration is increased. In dilute solution the collodion membrane and the onion epidermis are negatively charged. Pores in such membranes are therefore presumably lined with a fixed layer of adsorbed ions, principally anions, and the lumen of the pores if they are sufficiently small contain principally cations which can be displaced relatively to the fixed layer. This accounts for the relatively high apparent mobility of cations in the membranes. When the concentration is increased it may be supposed that the differential adsorption decreases; there will, therefore, be more cations in the fixed layer and more anions in the free layer, and if both ions negative and positive are univalent the potential across the membrane will approximate to the liquid junction potential as the number of cations and anions in the fixed layer, and consequently in the displaceable layer, becomes more and more nearly equal.

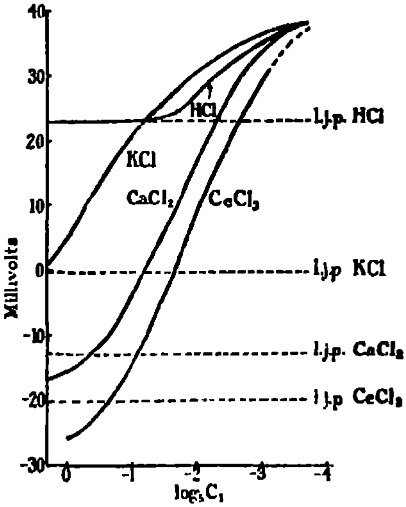

FIG. 4.

If the cation, however, is not univalent, then, when with increasing concentration the number of anions and cations in the fixed layer tends to equality, the ratio of anions to cations in the displaceable layer will tend to 2 for a bivalent and 3 for a trivalent cation and the membrane will behave as if relatively impermeable for cations.

This theory accounts qualitatively for the facts described above and for the behaviour of the collodion membrane and is in agreement with what is known of the discharge of non-ampholyte colloids such as a gold sol by electrolytes. Quantitative proof would require it to be shown that at the concentration of calcium chloride, for example, at which the potential across the membrane is equal to the liquid junction potential, the zeta potential of the membrane is zero. Unfortunately, direct cataphoretic measurements are equivocal, owing to the impossibility of obtaining a sufficiently high potential gradient with concentrated solutions without heating and convection.

Summary.

The inner epidermis of the onion scale is shown to resemble the dried collodion membrane in being relatively very impermeable for anions in dilute solutions of electrolytes and in retaining this impermeability for an indefinite period.

For uni-univalent electrolytes the potential across the membrane tends with increasing concentration to a limit equal to the liquid junction potential in the absence of the membrane. For electrolytes containing a bi- or trivalent cation the potential falls with increasing concentration below the value of the l.j.p. and the membrane appears to be more permeable for anions than for cations.

Addition of small amounts of bi- or trivalent cations to solutions of potassium chloride in contact with the membrane has a disproportionately large depressing effect on the potential.

This work was carried out while holding the Amy Mary Preston Read Scholarship at the University of Cambridge. I wish to express my gratitude to the Governing Body of Trinity Hall for financial help, and my sincere thanks to Mr. James Gray, F.R.S., for his continual interest and encouragement.

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548.73 : 547.458.81

X-ray Analysis of the Structure of the Wall of Valonia ventricosa.—I.

By W. T. ASTBURY and THORA C. MARWICK, Textile Physics Laboratory, University of Leeds, and J. D. BERNAL, Mineralogical Laboratory, University of Cambridge.

(Communicated by Sir William Bragg, F.R.S.—Received November 13, 1931.)

[PLATE 39.]

In searching for material built from cellulose which might, by the application of X-ray analytical methods, throw further light on the nature of the cellulose lattice and its orientation in biological structures, Sponsler (1930) examined the green alga, *Valonia ventricosa,* which grows in the form of a single-celled hollow sphere often as much as 2 cm. in diameter. From the X-ray photographs which he obtained, he was able to show, not only that the cell-wall is composed of crystallites of cellulose which for such a substance must be considered as relatively well-developed, but also that these crystallites are so laid down in the wall that there is a preferential orientation of the crystal lattice with respect to the surface. The plane of "spacing" 6·10 A.U. lies roughly parallel to the wall, while the plane of "spacing" 5·33 A.U. lies roughly perpendicular to the wall. On the other hand, Sponsler (1931) concluded that there is no further selective orientation of the cellulose crystallites, but that, "in attempting to determine, in *Valonia*, the position of the remaining axis, the (cellulose) chain axis, the diffraction lines which indicated the presence of the chains showed that a random distribution of the chains occurred around a radial reference line of the wall."

By the courtesy of the same worker, the actual specimens on which these observations were made were soon afterwards submitted to an optical examination in polarised light by R. D. Preston (1931) of the Botany Department of the University of Leeds. His results appeared to offer a striking confirmation of those of Sponsler, for he found that the wall was divided approximately into areas forming a mosaic, each area having its own interference colour, different from that of its surroundings. The average size of the areas was about $1 \cdot 5 \times 10^{-4}$ sq. cm., and the extinction directions in general varied considerably in passing from one area to another. Preston assumed, in accordance with what appears to be a well-established, but as the sequel shows erroneous, custom, that the extinction directions in any area were parallel and perpendicular to the directions of the cellulose chains in that area, and therefore concluded, since many interference patches would be irradiated simultaneously by an X-ray beam such as Sponsler used, that the effect of random orientation described by the latter was in perfect agreement with his own findings. The fact, however, that Sponsler had used for his investigations, not single pieces of cell-wall, but blocks made up of a large number of such pieces, suggested that there might be a fallacy in this argument, even though the blocks in question were so built up that meridians running from the holdfast of the spherical cell were as far as possible parallel. In spite of the fact that the optical examination suggested the contrary, it was still possible that the X-ray diffraction effects had been produced by the accumulation, in the large mass of material used, of relatively slow variations of orientation distributed over the whole surface of the cell-wall. It seemed, therefore, that a more promising line of attack would be to take X-ray photographs of *single* pieces of wall and, if possible, limit the X-ray beam each time to a single interference patch which had been previously defined with the aid of the polarising microscope.

Such a series of photographs, of the actual specimens of *Valonia* examined by Preston, have now shown that the second interpretation is in fact the correct one, and that the optical discontinuities are to be ascribed to quite a different cause from what was originally proposed. A single piece of cell-wall, though no more than a small fraction of a millimetre thick, gives a good X-ray photograph which is in general almost completely free from the diffraction effects to be expected from cellulose chains in random orientation about a normal to the wall (fig. 2, Plate 39). On the contrary, each photograph generally shows reflections from two principal sets of cellulose chains crossing each other at an angle somewhere near a right angle, with often traces of a third orientation lying about half-way between the other two. Furthermore, substantially the same X-ray photograph, showing practically the same orientations of fibres, was obtained when the exploring X-ray beam was moved through a distance of several millimetres over the surface of the cell-wall. In short, the first series of photographs demonstrated clearly that :—

(1) The wall of *Valonia ventricosa* is built up of two main sets of cellulose chains lying in the wall and crossing at an angle which is generally near about 80° but which may be as small as about 60° ;

(2) The cellulose chains are in the form of well-defined crystalline aggregates;

(3) The orientation of each set of crystallites thus formed is usually remarkably perfect;

(4) In the majority of areas examined the two sets of crystallites were in roughly equal proportions;

(5) There is frequently a small proportion of chains in another orientation lying between the two main orientations;

(6) The orientations of the various sets of chains were roughly constant over the area of the small pieces of cell-wall available for examination (about 5 mm. across);

(7) There are apparently changes of orientation of an, as yet, undefined character;

(8) The fibre orientations persist through the whole thickness of the cell-wall, since two lamellæ made by splitting a single piece of the wall gave similar X-ray photographs;

(9) As observed by Sponsler, there is a further selective orientation of the cellulose chains so that the plane of spacing 6·10 Å.U. tends to lie parallel to the cell-wall; but there is undoubtedly a dispersion of this plane, the actual value and variations of which are at the moment under investigation.

This X-ray investigation is now being continued by Preston (with the aid of a grant from the Department of Scientific and Industrial Research). His additional results to date serve to confirm the conclusions outlined above. They also show that the interference discontinuities previously described by him are of secondary importance, in the sense that they have no influence on the main features of the X-ray photographs and are not universally present in the pieces of *Valonia* examined. Our attention has thus been attracted once more to the very fine and often very faint striations which may be detected on the wall of *Valonia ventricosa*, and which appear to continue uninterruptedly over considerable distances, *whatever the respective interference colours of the areas which they traverse.* It has long been known that plant walls are not simply lamellated, but that they show surface striations, the first definite account of which is due to von Möhl (1836). During the last century the phenomenon gave rise to some discussion (Nägeli, 1866), but it seems that generally satisfactory conclusions were not arrived at concerning its precise nature. It is now clear that *in the wall of* Valonia ventricosa *the striæ are* *parallel to the directions of the cellulose chains as revealed by X-ray analysis.* In general, two sets of striæ may be detected which correspond exactly to the two main cellulose orientations described above. The whole wall is thus apparently either a kind of basket-work or a ply-wood structure of crossed cellulose fibres of probably submicroscopic dimensions.

Fig. 1 shows diagrammatically the experimental arrangement designed to demonstrate this point. A fragment of *Valonia* was mounted across a hole in a piece of brass, and a hair was mounted, under the microscope, across the *Valonia* so as to be approximately parallel to one of the two sets of striations. A photomicrograph was then taken of the striations and hair. A gate-like rectangle of wire was then attached to the brass so as to swing with its outer side always parallel to the hair and therefore to the striations. While an X-ray photograph was being taken of the area under examination, the outer side of the wire rectangle rested against the photographic film and so left a shadow parallel to the striations. A photomicrograph of the striæ and the corresponding X-ray photograph, both prepared by Preston, are shown in fig. 3, Plate 39. It will be seen that the cellulose chains, which lie perpendicular to the two main rows of spots in the X-ray photograph, correspond closely to the striæ observed under the microscope.

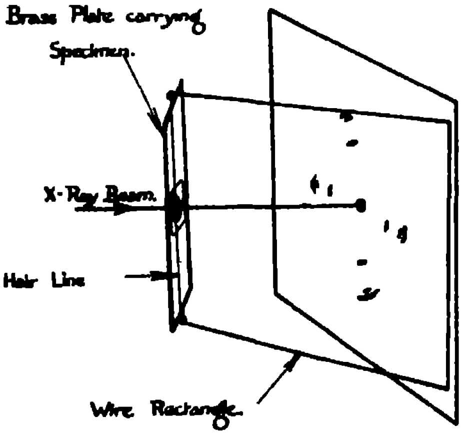


Fig. 1.

From this demonstration follows at once an important point with regard to the conclusions to be drawn from the examination of cell-walls in polarised light. *The extinction directions do not necessarily correspond to the directions of the cellulose chains.* In fibres such as ramie, where X-rays reveal only one set of cellulose chains lying practically parallel to the fibre length, there is

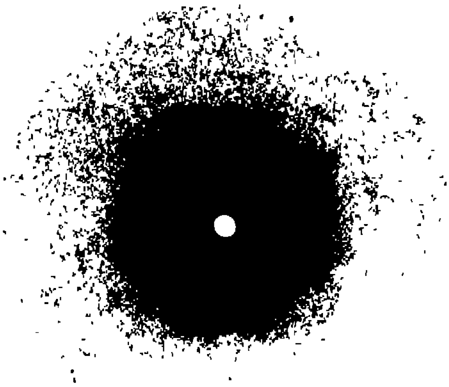
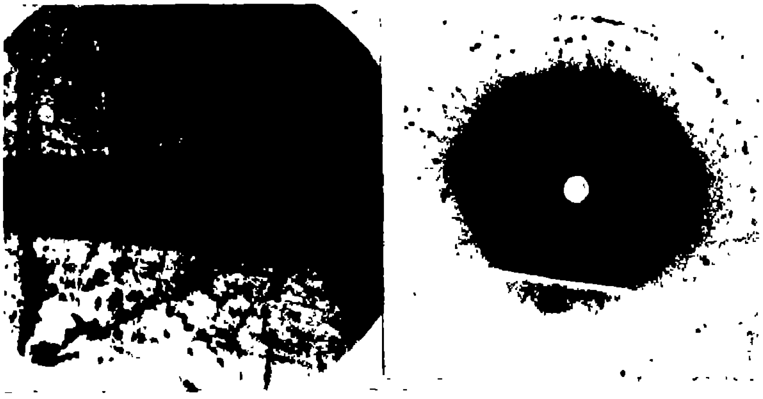


FIG. 2.—X-ray photograph of the wall of *Valonia ventricosa*, taken with the rays normal to the wall, and showing two main cellulose orientations inclined at 78° to each other, together with traces of a third intermediate orientation. Cu K rays. $d = 3$ cms.



(*a*) (*b*)

FIG. 3.—(*a*) Photomicrograph of the cell-wall of *Valonia ventricosa* showing two sets of striations. The dark bar is a hair. (*b*) X-ray photograph of the same area. The white shadow is a wire parallel to the hair shown in (*a*). The two rows of spots in the X-ray photograph are perpendicular to the cellulose chains, which are therefore parallel to the striations of the cell-wall. (Preston.)

naturally straight extinction; but when there are more than one set of chains, as in *Valonia*, the extinction directions lie between the two chain directions, as theory predicts. In pieces of *Valonia* free from interference patches the extinction directions exactly bisect the angle between the cellulose chains when X-rays show that the two orientations are in equal proportions. Fig. 2, Plate 39, is an X-ray photograph of such a piece which showed, except for secondary irregularities, perfectly uniform extinction, with the extinction directions lying half-way between the chain directions given by the X-ray photograph. In those pieces of *Valonia* in which X-rays revealed a definite difference between the proportions of the two chain orientations, the extinction, just as we should expect, was moved towards the orientation of greatest intensity. Thus, as far as we can see at the moment, the interference patches shown by certain regions of the wall of *Valonia ventricosa*, with their varying extinction directions as described by Preston, are most simply explained as due to varying proportions of the two sets of cellulose chains. Such an interpretation is in full agreement with all the facts at present known to us.

We are indebted to Dr. F. C. Phillips, of the Mineralogical Laboratory of the University of Cambridge, for a further optical examination of pieces of *Valonia* with uniform extinction. He has very kindly determined their refractive indices and optic axial angles with the aid of a Fedorow Universal Stage. His results are given in Table I (specimen II corresponding to fig. 2, Plate 39).

Table I.

Specimen.	I.	II.
Interfibre angle by X-rays	58°	78°
Birefringence	0·01	0·006
2V	83°	63°
γ	1·56–7	1·56
β	1·55–6	1·554
α (calculated)	1·541	1·545
γ (calculated for fibres all in one direction)	1·58	1·58

As would be expected, they show that the specimen built of chains inclined at the smaller angle has a greater birefringence and a greater optic axial angle than the specimen built from chains inclined at the larger angle; in fact, both the X-ray and optical examinations agree in indicating that considerable areas of the wall of *Valonia* closely resemble the structure of a finely repeated "twin." Such areas behave optically as single crystals, and their properties can be explained by the assumption of a large number of thin layers alternately of one orientation and then of the other. Actually it is possible to separate the wall into a number of thinner lamellæ (Preston, *loc. cit.*), as in the case of mica, and cross-sections under the microscope appear to show about as many as forty such layers; but it still remains to determine the limit of the lamellation. A complete description of the structure of the wall of *Valonia* must indicate whether the two orientations of cellulose chains alternate as we pass from one layer to the next, and if so, what is the limiting thickness of such a layer of uniform orientation, or whether there is an intimate admixture of the two orientations throughout the whole thickness of the wall. The evidence as yet available does not settle his point—an important one from the point of view of the mechanism of growth—but we are investigating the matter further.

The γ refractive index (1·58) calculated for fibres all in one direction is rather smaller than the mean value found by Frey (1925–26), who states that for fibres such as ramie the cellulose micelles are practically uniaxial positive, with $n_\gamma = 1{\cdot}594$ and $n_\beta \simeq n_\alpha = 1{\cdot}533$, n_γ corresponding to the long axis of the micelles. With interfibre angles of 58° and 78°, and assuming equal proportions of the two sets of fibres, Frey's values would give :—

$$\left.\begin{array}{l}\gamma_{\text{Valonia}} = 1{\cdot}576\\ \beta_{\text{Valonia}} = 1{\cdot}546\end{array}\right\}58^\circ \text{ and } \qquad \left.\begin{array}{l}1{\cdot}567\\ 1{\cdot}555\end{array}\right\}78^\circ.$$

The fact that Phillips' experimental results lead to a calculated γ which is too small and a calculated α which is too large is probably to be ascribed to a lack of strict parallelism between the chains and the cell-wall, possibly, in part, on account of a certain amount of folding in the wall.

It would appear that even these preliminary results of the application of X-ray methods to the problem of the structure of the wall of *Valonia ventricosa* are of so striking a character that they open up new and profitable fields in which we may hope that close co-operation between botanist and physicist will ultimately reveal the mechanism of the growth of cellulose in the living plant. As a next step in these researches we propose now to examine by X-rays a complete *Valonia* cell with a view to determining both the cellulose orientations in every part of the wall and the relative proportions of the cellulose chains which are allotted to the various orientations. When the latter are plotted on the surface of a sphere, it is possible that the resulting configuration viewed as a whole will throw light on the nature of the orienting forces. When the X-ray photographs described in this paper were first obtained, it was thought that the two main orientations might turn out to be based on crossed "twins" of the cellulose crystallites, that is, that the orienting forces in the cell-wall were of a familiar crystallographic nature; but the lack of strict constancy in the interfibre angle invalidates this suggestion. The important task at the moment seems to be to determine the limiting thickness of cell-wall associated with each orientation. In structures built of a single cellulose orientation, such as the cell-wall of ramie, the hypothesis that the orienting forces are crystallographic is certainly plausible; but in structures such as that of *Valonia*, in which we find two or more sharply defined orientations of *crossed* cellulose crystallites, it is not easy to see how the orientation of any crystallite is transmitted, not to its immediate neighbour, but to the next but one. As just mentioned, the mechanism of "twinning" would afford a ready explanation if the interfibre angle were constant; but this is not so. As it is, the experimental data at present available appear to suggest that we must look for something *outside* the crystals themselves as the origin of those forces which orient the cellulose fibres so perfectly.

Summary.

(1) The cell-wall of *Valonia ventricosa* is found by X-ray methods to be built up of two main sets of cellulose chains which form crystallites crossing at an angle which is maintained remarkably constant through the whole thickness and over considerable areas of the wall.

(2) The orientations of the cellulose chains are found to be parallel to the directions of the fine crossed striæ which may be detected on the surface of the wall when examined under the microscope.

(3) The extinction directions shown by the wall in polarised light are determined by the interfibre angle and the proportions of cellulose chains associated with each orientation.

(4) Refractive indices and optic axial angles have been measured for those areas of the wall which behave optically as single crystals.

(5) Suggestions are discussed as to the nature of the orienting forces.

In conclusion, we should like to express our thanks to Professor J. H. Priestley, of the Botany Department of the University of Leeds, for his encouraging interest in the work and frequent advice on the botanical aspects of the problems involved. The expenses of the investigation are being defrayed by grants from the Worshipful Company of Clothworkers, the Government

Grant Committee of the Royal Society, and the Department of Scientific and Industrial Research.

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Experiments upon Respiration in the Larvæ of certain Parasitic Hymenoptera.

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Introduction.

The method by which an apneustic endoparasitic insect larva obtains its oxygen and eliminates the carbon dioxide produced has long been a subject for speculation.

Many parasitic larvæ are characterised by the possession of long "tails," or other expansions of the body wall, and the tendency in the past has been to ascribe to such structures, whether in parasitic or aquatic forms, the function of gills; tracheal gills if they contain tracheæ; blood gills if they do not. Direct evidence as to the function of such structures is scarce, even concerning aquatic forms, and is almost completely lacking with regard to parasites. The work here described was undertaken in the hope of throwing some light on the matter with special reference to the two most striking instances, namely the "tail" of young endoparasitic Ichneumonid larvæ and the "caudal vesicle" of the larval Braconids. The results of similar experiments on a remarkable Dipterous parasite, *Cryptochætum iceryæ*, have already been published (Thorpe, 1930). It is hardly necessary to discuss the previous work on the subject and its relation to more general problems, since Wigglesworth (1931) has recently published an excellent summary of the literature dealing with insect respiration.

The study of the problems of parasite respiration is rendered difficult by lack of knowledge regarding the physiology of non-parasitic forms. For instance, there are one or two well-authenticated instances (Davis and Slater, 1928; Cole, 1921) of insects which, while normally aerobes can, under adverse conditions, subsist anaerobically for a considerable length of time and this ability may well be more widespread than is at present suspected. How far it occurs among parasitic larvæ is unknown, but the existence, especially among the later stages of many Tachinids, of an elaborate apparatus for securing communication with the atmospheric air and, in many of the Hymenopterous forms, of a highly developed plexus of fine tracheæ immediately beneath the hypodermis, gives a strong *a priori* reason for supposing that they are true aerobes. In the latter case, the greater part of their oxygen requirements is obtained by absorption of the gas by diffusion from a surrounding fluid containing it in solution. In other words, it seems probable that, in the parasitic Hymenoptera, the general body surface acts as a respiratory surface and that the problem of respiration in apneustic aquatic and parasitic larvæ is essentially the same. Another striking parallel between the respiration of parasitic and of aquatic forms is seen in the fact that in the first instar of many Hymenopterous parasites the tracheæ are either absent, or if present are filled with fluid and are non-functional, the gaseous exchange taking place directly between the tissue fluids of parasite and host.

Methods.

The study of respiration of aquatic larvæ is relatively simple. That of parasitic forms is greatly complicated by the fact that the experiments must, at best, be carried out under highly artificial conditions.

As the problem is to determine not the total respiration rate, but the relative rates of gaseous exchange taking place at different parts of the body surface, the only feasible method seemed to be the use of chemical and biological indicators somewhat on the lines of Fox's (1921) work on *Chironomus*. Parasitic larvæ were dissected out of the host, rapidly washed free of the host's blood and tissues, in a few drops of normal salt or Ringer's solution, and placed on a microscope slide under a coverslip raised on wax feet. For the study of carbon dioxide output a suitable p_H indicator solution is then run in under the coverslip, the colour change being observed under the binocular microscope against an opaque white background. The larvæ can be held motionless by slight pressure on the coverslip, although with the majority of parasite larvæ this is not necessary. In the case of more active larvæ it is usually possible to prevent movement by previous narcotisation with 1 to 2 per cent. urethane solution. The exact concentration required must be determined for each individual case.

The indicator must be one having a range somewhere in the region of neutrality and which is non-toxic at the concentrations necessary for the colour change to be easily visible in the thin layer under the coverslip. The indicator found most useful was cresol red. O-Chlorophenol indophenol at a strength of 0·1 to 0·5 per cent. was also employed. It has the advantages of being directly soluble in water, giving a very intense colour in dilute solutions with an easily visible colour change from blue (alkaline) to red (acid) between p_H 6·0 and p_H 7·0. In addition, phenol red and brom-cresol purple were used occasionally. The indicators were used in solutions of known p_H between 8·3 and 7·3 obtained by using different concentrations of Na_2CO_3 between 0·0025 N and 0·00025 N (Saunders, 1923).

An attempt was made to obtain quantitative results by plotting the area of colour change obtained around a respiring larva by means of a camera lucida and then calculating from the volume of fluid undergoing the change the total putput of CO_2 in a given time. It was found in practice, however, that the line of demarcation between the changed and unchanged indicator was so indefinite that it was impossible to determine with sufficient accuracy the outer limits of the area and consequently the results were unreliable.

In the case of larger forms a solution of barium hydroxide was also used to demonstrate carbon dioxide output, but with smaller larvæ this method is unsatisfactory owing to the difficulty of seeing very minute quantities of the white precipitate.

For studying the absorption of oxygen the biological indicator method was employed. As is well known, this consists in making use of the fact that certain organisms, especially flagellate protozoa, migrate to a region having a certain optimum oxygen tension lower than that in water saturated at atmospheric partial pressure. When a culture of a suitable organism is run in under a raised coverslip the organisms will at once form aggregations at those surfaces of the larva where oxygen is being absorbed from the fluid. As the tension of oxygen falls below the optimum, the flagellates will retreat from this region, leaving a clear space and moving outwards in crescentic formation as a retreating band with the respiring surface as its centre, till they come to lie as a narrow strip close to edges of the coverslip.

This method was first used for the study of the respiration of certain aquatic insects by Fox (1921), *Bodo* being the flagellate employed. Fox obtained his material from grass infusions in tap water and at first the present writer used the same method, the forms thus obtained being *Chilomonas*, *Paramœcium* and *Polytoma* sp. nr. *uvella*. The cultures obtained from infusions were, however, found to be unsatisfactory in various ways. They were, of course, never pure cultures and, after the fourth or fifth day, were usually highly contaminated with bacteria which hinder the flagellates from forming their usual clear bands. Thus it was impossible to ensure that any two experiments were being performed under identical conditions since no two cultures were identical in composition.

In order to enable the experiments to be carried out under properly controlled conditions, it was clear that pure cultures of a single organism would have to be used. A number of different organisms were tested, including *Polytoma uvella*, *Colpidium colpoda*, *Gonium*, and various motile bacteria, *B. mesentericus vulgatus*, *B. mycoides* (Hewlett), *B. aroideæ*, *B. subtilis*, Hoy. Of these *Polytoma* was found to be the most satisfactory. It was grown in pure culture in a glycocoll medium of the following composition :—

Sodium acetate	0·2 gm.
Glycocoll (amino-acetic acid)	0·2 gm.
Glucose	0·2 gm.
K_2CO_3	0·5 gm.
$MgSO_4$	0·01 gm.
K_2HPO_4	0·02 gm.
Water	100 c.c.

The medium, adjusted to the desired p_H, was placed in sterile test tubes, preferably of hard glass, and sterilised in the steamer in the usual way. Sub-culturing was carried out by means of long sterile pipettes of hard glass. In this way stock cultures were kept going satisfactorily for eighteen months, sub-culturing being necessary about every 20 days. When required for use in the experiments special sub-cultures were made from stock.

Polytoma grown in this way go through a regular cycle of changes during which they vary in their activity and rate of reaction to stimuli. They attain their maximum density about the fourth or fifth day. It was found that aggregation and banding take place with greater speed and intensity when the culture is from 48 to 72 hours old and consequently only cultures of this age were used in the experiments.

It was found possible to grow *Polytoma* satisfactorily at a considerable range of p_H. They will live and behave normally at any p_H between 5·6 and 9·4.

An acidity greater than p_H 5·2 is rapidly fatal. No attempt was made to grow them at a p_H higher than 9·4. Owing to the buffering of the fluid the p_H is found to remain approximately constant throughout the life of a culture. Cultures at any p_H within the range above mentioned are quite satisfactory for use, but increased alkalinity of the medium, up to about 8·8 or 9·0, tends to increase somewhat the activity of the flagellate and consequently the rate of banding also. Thus a culture at p_H 9·2 will have formed a band at the edge of the coverslip in 15 minutes, whereas at p_H 8·0 the same culture will require 35 minutes, and at p_H 7·2 and 6·0, 70 minutes will elapse before the same stage is reached. If the results are to be comparable it is necessary, therefore, always to use a medium of the same p_H.

In the case of *Bodo*, Fox showed that the spontaneous aggregation of the organisms was the result of active orientation of the flagellates to a specific oxygen tension and not to a specific hydrogen ion concentration. It is, however, well known that in certain protozoa, *e.g.*, *Spirostomum* (Saunders, 1924), as well as spermatozoa of *Nereis* and other organisms (Gray, 1922), which show similar aggregations, p_H due to dissolved CO_2, and not oxygen tension, is the dominating factor. It was, therefore, necessary to determine which of these two factors was the dominating one in the case of *Polytoma*. The fact that the flagellate while in a buffered medium behaves in an identical manner to *Bodo* in the various experiments described by Fox, suggests at once that O_2 tension and not p_H is the factor involved. This is confirmed by the following experiments. When a culture of *Polytoma*, at p_H 9·0, coloured with thymol blue, is allowed to band under the coverslip in the usual way, no difference in p_H can be detected between the inside and the outside of the band, till long after the band has reached its final position. If then the banding is due to a p_H reaction the optimum must be so little below p_H 9·0 that the difference is not distinguishable colorimetrically. But yet the flagellate will also form aggregations and bands in fluid at any p_H above 6·0. Again, a culture of *Polytoma* in glycocoll medium at p_H 9·0 and some fresh medium at p_H 8·0 are run in simultaneously at opposite ends of a long coverslip raised on wax feet. If the aggregation was due to preference for a region of p_H lower than 9·0 then a dense band should form immediately where the two fluids meet. This does not happen; the mode of aggregation and the subsequent banding being identical with that which takes place in a control experiment in which the culture fluid and the fresh medium are at the same p_H. The behaviour of the flagellates in light and in darkness appears to be identical.

It will be seen that there are other advantages in using pure cultures in a medium of known and constant composition besides the ones mentioned above. By this method the necessity for filtering the culture through bolting silk is avoided, it merely being necessary to bubble air through the culture before use to ensure saturation with oxygen, and the use of a uniform culture fluid eliminates variation in behaviour due to differences in nutrition or food requirements. Finally, immersion in the glycocoll medium does not appear to have any injurious effect on even the most delicate parasite larvæ.

When required for use, a few cubic centimetres of fluid are pipetted off from the top of the fluid in the tube and transferred to a watch glass. All glass used must be scrupulously clean. The larva to be used in the experiment should be washed first in a few drops of the culture fluid. Since the flagellates are themselves absorbing oxygen they will, of course, in time go through the same manœuvres even though no larva be present under the coverslip, but in this case the aggregations will begin simultaneously at various points after a much greater lapse of time. A control experiment, in which no larva is present under the coverslip, must, therefore, always be started at the same time, and if aggregations at the surface of the larva do not begin at an appreciable time before they occur in the control the results must be considered as untrustworthy. Other control experiments were performed frequently throughout the course of the work. Thus larvæ killed by heat or other means always failed to give results. As a further test the various methods were also employed frequently on a variety of aquatic larvæ and were found to give consistent results; the respiratory function of the tracheal gills of *Ephemerids* and *Gyrinids*, for instance, being demonstrated in a most striking manner. An account of these experiments will be published later.

As a further test for oxygen consumption, the method of using a culture of luminous bacteria, first suggested by Beijerinck (see Kostka (1929)), was employed with considerable success. This method consists in taking advantage of the fact that the luciferin-luciferase action which results in the production of light can only take place in the presence of oxygen. The test has been shown to be exceedingly delicate, since the light is produced in concentrations of oxygen as low as 0·0007 per cent.

The stock of *B. phosphorescens* was grown in pure culture on slants of salt agar. When required for use cultures in a liquid medium were set up. The medium used consists of a fish broth with the addition of 0·5 per cent. glyceroll, 0·5 per cent. peptone, and 3 per cent. NaCl, adjusted to a p_H of 8·0. For use the fluid culture is diluted with 1–2 volumes of distilled water, aerated, and run in under the coverslip in the same way as the *Polytoma* cultures, save that the experiment is carried out in the dark room and all manipulations performed by the light of a dark-room red lamp. The experiment must be watched closely under a low power of the binocular microscope in complete darkness. The insect, of course, appears as a black area in the luminous fluid. As soon as the oxygen tension is reduced to a certain point the light begins to fade and a black area spreads gradually outwards from the respiring larva. Eventually the whole of the field will become black with the exception of a narrow band around the edge which continues to shine with undiminished brilliance. During the course of the experiment a dull red light can be used as a microscope lamp, this being switched on momentarily so that the exact position of the animal in the growing dark area can be seen and the exact width of the dark band at different regions of the body can be determined. Since the bacteria themselves are using up oxygen, a control must be run simultaneously with the experiment as with *Polytoma*.

This method was found to be very successful with the larger organisms and was used frequently. With the smaller forms it is more difficult to work satisfactorily, although even with these it served as a useful confirmatory test of the results obtained with *Polytoma*. Its chief advantage lies in the fact that it is absolutely specific for oxygen, effects due to varying p_H being completely eliminated. On the other hand, it has its various drawbacks, the chief of these being the difficulty of manipulation of small larvæ in semi-darkness and the length of time required for the work. Not only does each experiment take from one-half to three-quarters of an hour, but from one-half to three-quarters of an hour, at least, must be spent in a subdued light and some 15 minutes in absolute darkness before the eyes become sufficiently accustomed to carry out really accurate observations. Another disadvantage is that the bacteria can only be grown in a medium of high salt concentration which, even at the maximum dilution possible for accurate experiment, is rapidly fatal to more delicate larvæ such as *Apanteles*.

Caudate Ichneumonid Larvæ.

Caudate larvæ are known to occur in a variety of parasitic Hymenoptera. They have been recorded as present in the *Ichneumonidæ*, *Braconidæ*, *Chalcidæ*, and *Cynipidæ*. In every case the " tail " is at its maximum development in the first instar, and is progressively reduced in the later stages. This organ is seen at its largest in the *Ichneumonidæ*, and it is with Ichneumonid larvæ that the experiments were carried out. The structure of this appendage is shown in figs. 1 to 3 and 6 to 7.

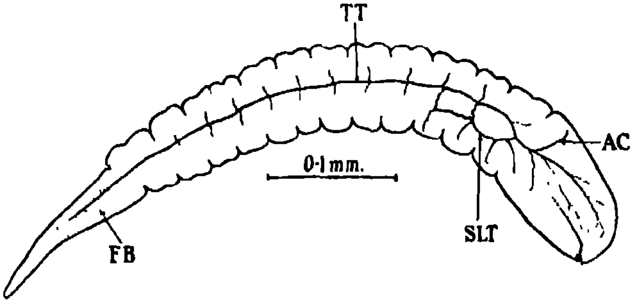

FIG. 1.—First stage larva of *Angitia nana*, to illustrate the type of tail usually found in the *Ichneumonidæ*. The tracheal system of the right side only is shown. TT = tracheal trunk. A.C. = anterior commissure. SLT = secondary lateral trunk. FB = fat body.

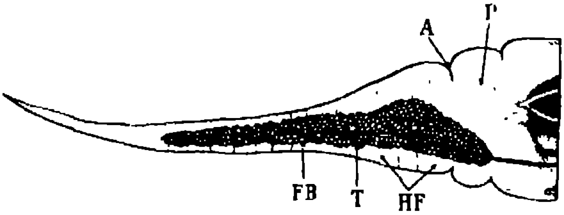

FIG. 2.—Longitudinal section of tail of *Eulimneria crassifemur* to show the structure characteristic of this type of tail. (Redrawn from Thompson and Parker.) Lettering as above. HF = hypodermal fibres. A = anus. P = proctodæum.

As regards Ichneumonid larvæ, the following are the chief theories which have been advanced as to the function of the tail. Seurat (1899) described it as a locomotive organ, Weissenberg (1909) concluded that it was an organ for the storage of waste matter. According to Timberlake (1912) and others it must be regarded as a blood gill, and according to Tothill (1922) a tracheal gill, this last supposition having also been put forward by Ratzeburg and others as far back as 1844. The observations of Muesebeck (1918) give support to

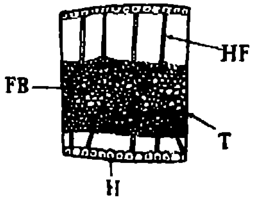

FIG. 3.—Longitudinal section of tail of *Eulimneria crassifemur* to show tracheal ending typical of such forms. (Redrawn from Thompson and Parker.) Lettering as above. H = hypodermis.

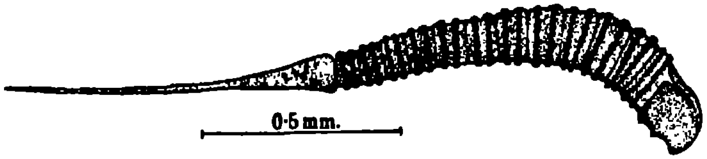

FIG. 4.—First stage larva of *Cremastus interruptor*, to illustrate a less frequent type in which the tail is relatively much larger.

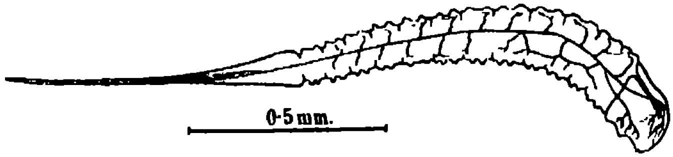

FIG. 5.—First stage larva of *Cremastus interruptor* to show tracheal system of right side.

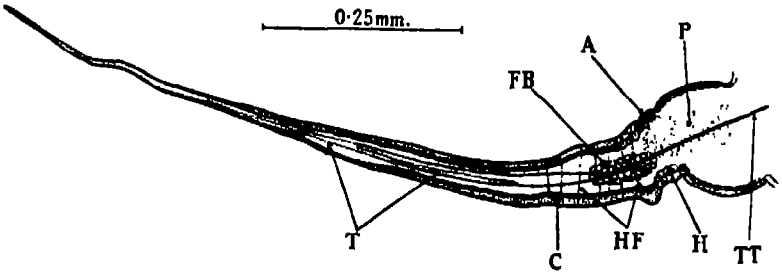

FIG. 6.—Diagrammatic longitudinal section of tail of *Cremastus interruptor* to show mode of ending of tracheal trunk of right side. The three fine tracheal branches extending throughout the basal two-thirds of the tail are an unusual feature. Lettering as in figs. 1–3. C = chitinous cuticle.

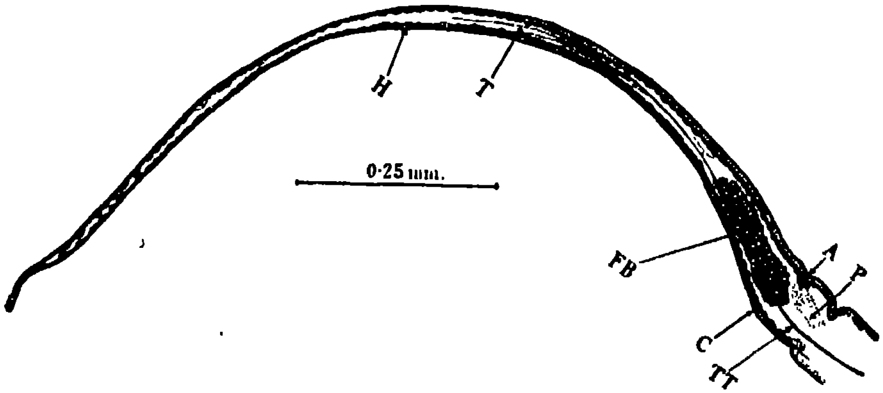

FIG. 7.—Similar tail found in *Omorgus mutabilis*, but with a less extensive tracheal supply. Lettering as in fig. 6.

Seurat's suggestion as regards certain *Braconidæ*, but it seems very doubtful how far it can apply to the *Ichneumonidæ*. In the majority of cases, first stage Ichneumonid larvæ seem to be capable of very little movement, and moreover, the tail is practically, if not entirely, devoid of muscles. Weissenberg's theory appears to have no more evidence to support it than that of Seurat.

With regard to the blood gill supposition, it is obvious that in order to be an efficient blood gill an organ must have a good blood circulation. This does not exist in any of the larvæ that I have observed nor, as far as I can discover, in any that have been described. The theory that the tail is a tracheal gill rested mainly on Tothill's description of an extremely rich supply of tracheæ supplying the cells within this organ, but Thompson and Parker (1930) have shown these observations to be erroneous. In the majority of forms the tracheal supply in the tails is confined to the two main tracheal trunks which terminate in the fat body in the base of the organ (fig. 3). The somewhat greater tracheal supply found in *C. interruptor* (fig. 6) appears quite exceptional.

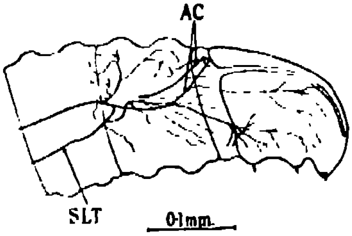

FIG. 8.—Tracheal supply of right side of head and first segment of *Cremastus interruptor*. Lettering as in fig. 1.

The suggestion put forward by Ayers (1884) that the tail and embryonic appendages found in the primary larvæ of many *Proctotrypids* and *Cynipids* are respiratory in function appears hardly worthy of serious consideration. That such minute larvæ should need special respiratory organs is hardly credible.

Experiments with Caudate Ichneumonid Larvæ.

The following larvæ were used for experimental purposes :—

Parasite.	Host.		Experiments.
Campoplex phthorimeæ Cushman (sp. ?).	*Phthorimea operculella*, Zell.	..	12
Cremastus interruptor, Grav.	*Rhyacionia buoliana*, Schiff.	..	26
Omorgus mutabilis, Holmg. ..	*Rhyacionia buoliana*, Schiff.	..	6
	Total	....	44

As regards all these larvæ the experiments gave no indication that the tail is of importance as a respiratory organ, either for the absorption of O_2 or the elimination of CO_2. Figs. 9, A–D, give typical examples of band formation. In the majority of experiments the tail produced no reaction at all. In only one case, an experiment with *O. interruptor*, was there any visible reaction of the flagellates to the tail itself, and then only at the base and this was quite subsidiary to the reaction at the surface of the posterior body region. As shown in fig. 9, the aggregations were usually first noticeable on the ventral surface of the thoracic segments, later appearing dorsally and on the ventral surface of the posterior abdominal segments. No sign of respiration was visible at the head.

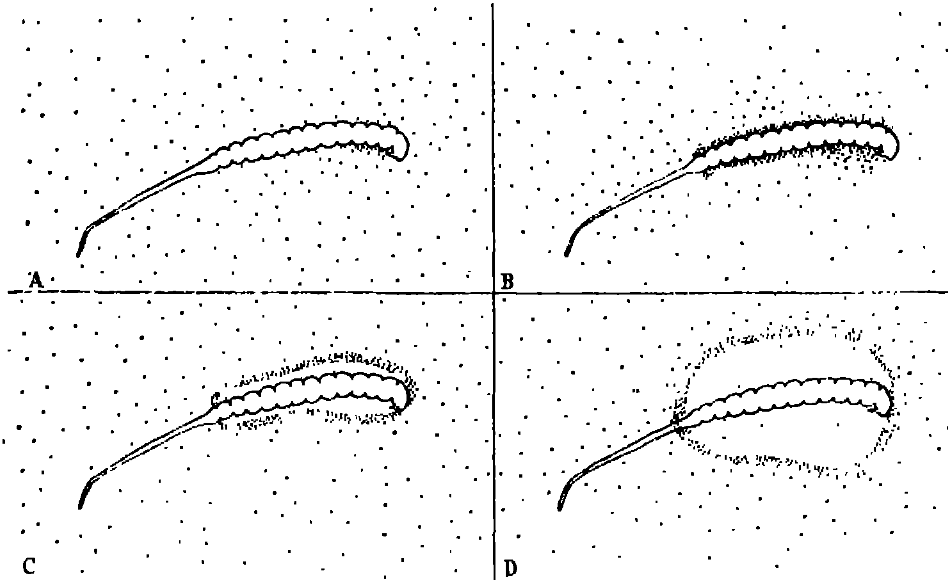

Fig. 9.—Aggregations and bands formed by 48-hour culture of *Polytoma* with first stage larva of *Omorgus mutabilis*, selected as a typical example of the experiments with tailed Ichneumonid larvæ A, 6 minutes; B, 7 minutes; C, 10 minutes; D, 15 minutes. Corresponding stages in the control experiment were reached 5 minutes later.

In the indicator experiments, the first colour change was also always to be seen at the ventral thoracic surface, later spreading over the body. Eventually a slight change could usually be observed at the base of the tail, but it always was quite secondary and often absent.

Similar experiments with the later stage larvæ in which the tail is very much reduced or completely atrophied always gave a general body surface reaction, there being no evidence of localisation of respiration.

Vesicle-bearing Braconid Larvæ.

In a number of Braconids the second stage larva (fig. 10) is provided with a bladder-like organ at the posterior end of the body formed by the evaginated proctodæum. It is withdrawn towards the end of the last instar when the larva leaves its host and spins a cocoon externally. This organ is known to occur in the following subfamilies, Microgasterine (*Microgaster*, *Apanteles*), Agathinæ (*Orgilus*), and Cheloninæ (*Ascogaster*), while a somewhat similar eversion of the rectum is known in the Macrocentrinæ. In addition, what appears to be a homologous structure is described in the aberrant genus *Banchus*, now placed among the Ophionine Ichneumonids.

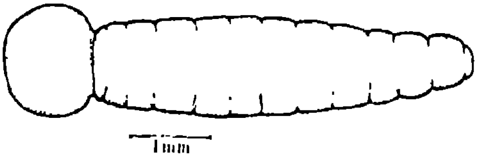

Fig. 10.—Late second stage larva of *Apanteles*.

Since the time of Ratzeburg there has been much rather fruitless speculation as to the function of this structure. It is not necessary to discuss this in detail; suffice it to say that opinion has been fairly evenly divided between the hypothesis that it is a blood gill and the supposition that it is primarily an excretory organ. The gill supposition has much more to support it in this case than in that of the caudate larvæ, since in the only forms (*Apanteles*, *Microgaster*) which have hitherto been thoroughly investigated a good blood circulation is present. Moreover, the larvæ are of a size such that a special respiratory organ might be deemed necessary.

Grandori (1911), who was the first to make a detailed investigation of the structure from an anatomical and histological point of view, showed that in *Apanteles* the greater part of the cavity of the vesicle is actually filled by the posterior chamber of the dorsal vessel. From this fact he favours the blood gill hypothesis, but on histological grounds he suggests that it has also an excretory function. Finally, since he describes the fore gut as closed in the species dealt with, he is constrained to suggest yet another rôle for the vesicle, namely that of food absorption.

However, the extent to which the apparatus varies as regards structural detail in the different genera has not hitherto been realised. It is accordingly unsafe to generalise as to function from examination of, or experiment upon, one type only.

Figs. 11 to 13 show in diagrammatic form the differences in structure between the three main types.

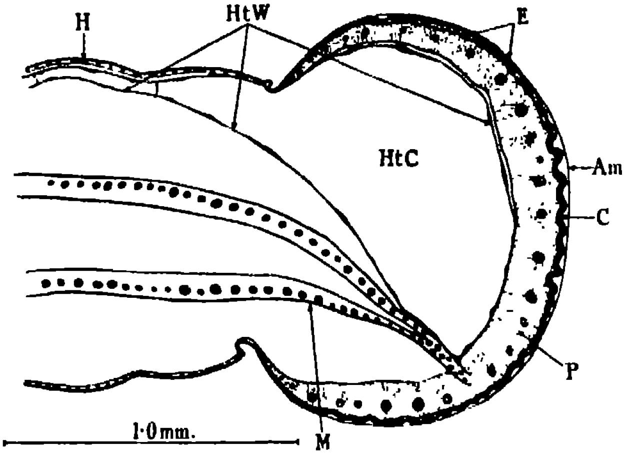

FIG. 11.—Diagrammatic longitudinal section of anal vesicle of *Apanteles glomeratus*. (Partly after Grandori.) HtC = cavity of heart. HtW = wall of heart. H = hypodermis. M = mesenteron. P = proctodæum. C = chitinous cuticle. Am = outer membrane formed from the persistent amnion. E = "excretory droplets."

Experiments with Vesicle-bearing Larvæ.

The following larvæ were used for experimental purposes :—

	Parasite.	Host.	No. of experiments.
Type 1.	*Microgaster* sp. (*congregatiformis*, Vier. ?)	*Phlyctænia ferrugalis*, Hubn.	24
	Ascogaster quadridentatus, Wesm.	*Cydia pomonella*, L.	2
	Apanteles popularis, Hal. ..	*Tyria jacobaeæ*, L.........	36
Type 2.	*Orgilus obscurator*, Nees.....	*Rhyacionia buoliana*, Schiff.	25
		Total	87

As regards the oxygen absorption of type 1, the results are illustrated by fig. 14, A–F (p. 465). There appeared at first to be a good deal of individual variation, in some individuals the vesicle apparently having less respiratory function

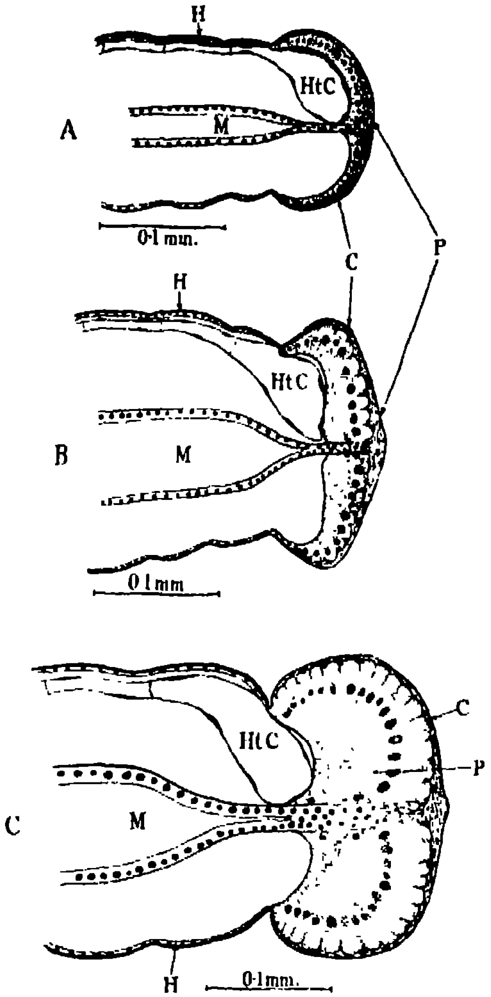

Fig. 12.—Diagrammatic longitudinal section of anal vesicle of *Orgilus obscurator* to show reduction of cavity due to increasing thickness of proctodæum. Note absence of outer amniotic membrane. A = first instar. B = early second instar. C = late second instar. Lettering as fig. 11.

than the rest of the body. This was found to be due to the fact that larvæ of this type are very delicate and that manipulation sometimes causes cessation of the heart beat within the vesicle and partial withdrawal of the vesicle itself. The results of experiments in which this happened had therefore to be discarded. The illustrations are taken from experiments in which the heart and vesicle were acting normally throughout, and show fairly uniform results. Aggregations were usually first observable at the posterior region of the body, becoming noticeable at the vesicle and at the rest of the body surface some 20 to 30 seconds later. These aggregations at the general body surface are especially noticeable in the case of *Apanteles popularis*, which has a very dense subcuticular network of fine tracheæ over the whole body.

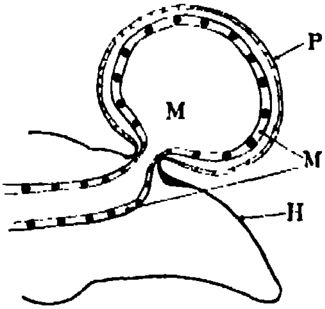

Fig. 13.—Diagram of vesicle structure in *Banchus femoralis*. (Redrawn from Bledowski and Krainska.) Lettering as fig. 11.

Table I.—Showing time in minutes taken to reach given stage of band formation in five experiments with second stage larvæ of *Apanteles popularis*.

Stage of band formation (see fig. 14).	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.	Control.
A	3½	1	2½	1½	1½	10–12*
B	6	2½	4½	3	2½	—
C	7	5	6	6	5	—
D	11½	10	7½	8	11½	—
E	14	15	12	11	14½	—
F	18	20	16	16½	18	30

* Time at which first aggregation observed.

Table II.—Showing time in minutes taken to reach given stage of band formation in six experiments with *Orgilus obscurator* with second or late first stage larvæ.

Stage of band formation (see fig. 15).	Experiment 1.	Control.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.	Experiment 6.	Control.
A	2	14*	4	4	3	2	3	16*
B	3	—	10	7	6½	5	5	—
C	12	—	15	13½	11½	8	7½	—
D	17	—	25	16	15	12	11½	—
E	22	30	30	20	18	17	16	35

* Time at which first aggregation observed.

In the case of type 2, a somewhat different result was obtained, as might be expected in view of the differences in structure and relative size. When, as in the early second instar, the vesicle is small it appears to have no respiratory function whatever. In the later second stage, though larger, the blood flow is still very imperfect and the proctodeal epithelium very thick compared to

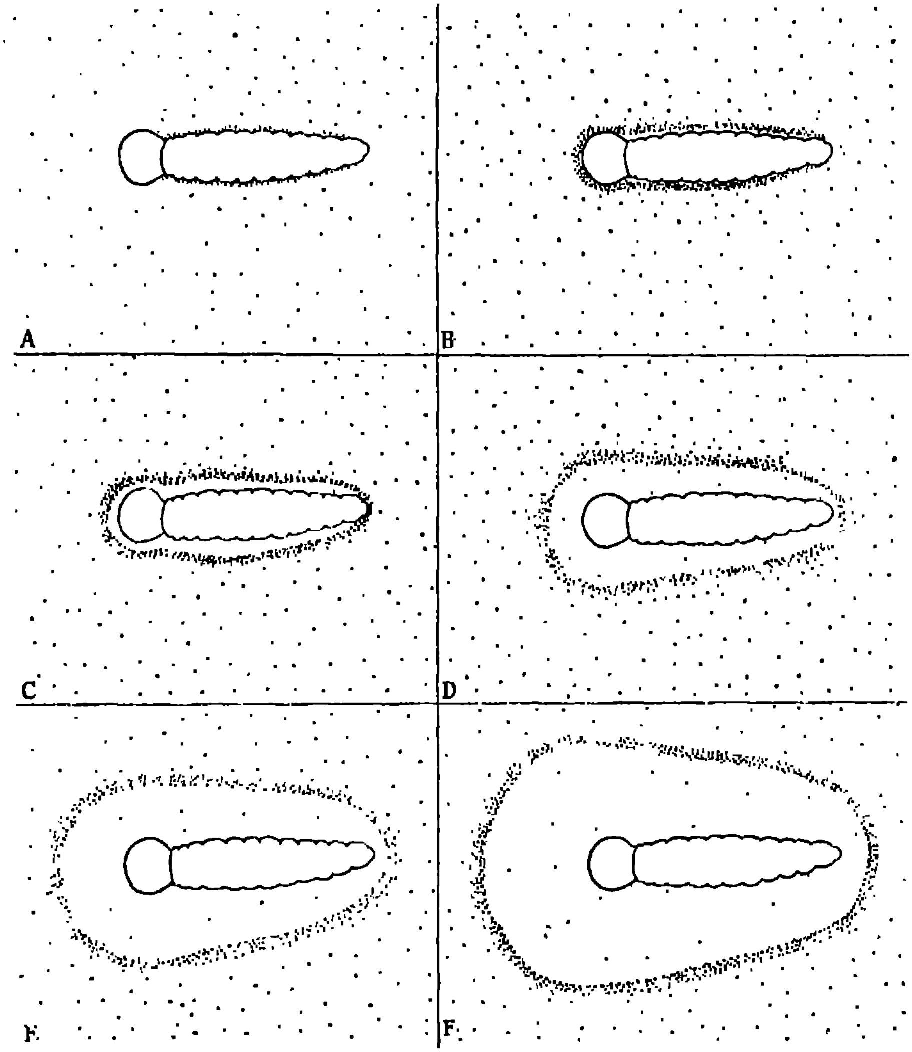

Fig. 14.—Characteristic *Polytoma* band formation with late second stage larva of *Apanteles popularis*. A, 1 minute; B, 2½ minutes; C, 5 minutes; D, 10 minutes; E, 15 minutes; F, 20 minutes.

that seen in *Apanteles* (fig. 15). As fig. 15 indicates, even when the vesicle is at its maximum development it is of comparatively little importance compared to the rest of the body surface, although respiration is proceeding rather more rapidly at the vesicle than at the head region.

Experiments with indicators completely confirmed the results obtained by means of *Polytoma* cultures. In other words, as in the case of the caudate larvæ, there appears to be no significant difference between the methods of oxygen absorption and carbon dioxide elimination. Special care is, however, necessary in the use of chemical indicators on larvæ of the vesicle-bearing type on account of their sensitiveness to injury and to unfavourable experimental conditions; otherwise the results may be quite misleading. Thus it was found necessary to use the indicators in concentrations as weak as possible and dissolved in isotonic solutions, otherwise the contraction of the posterior heart chamber is stopped and the proctodeal layer begins to be withdrawn. The vesicular cuticle is penetrated immediately by a great variety of dyes, the proctodeal layer taking up stains such as methylene blue, carmine, o-chlorophenol indophenol, and various p_H indicators within a few seconds. This sensitiveness rendered the use of the luminescent bacteria method unreliable in this particular case, nor could barium hydroxide solution be employed satisfactorily. This latter reagent, however, while it disorganises the vesicle immediately, leaves the body of the insect unaffected for a considerable time and with it the activity of the subcuticular network of tracheæ in the elimination of CO_2 could be demonstrated under a moderately high power of the microscope in a most striking manner. In the case of *Apanteles* the amnion persists as a delicate membrane enclosing the vesicle (fig. 11). This is highly permeable to dissolved salts with the result that the fluid within it rapidly assumes the same p_H as the salt or Ringer's solution

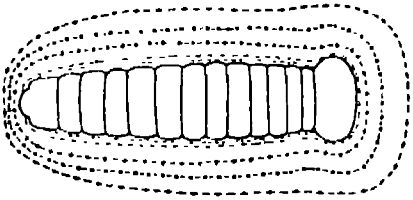

FIG. 15.—Diagram to illustrate band formation with *Orgilus obscurator*, a form with a less efficient type of vesicle. Dotted lines illustrate positions reached by bands at the following times. — — — — — A, 2 minutes. —·—·—. B, 4½ minutes. +—+—+— C, 7 minutes. —o—o—o D, 9 minutes. ·●· — ·●· — E, 12 minutes.

into which the larva is first dissected. Consequently the larva must be left in a few drops of the indicator fluid for a short while before an experiment is begun, otherwise the results will be unreliable.

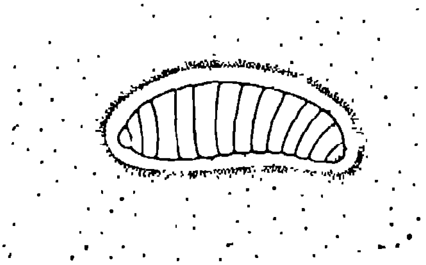

FIG. 16.—Type of band formations obtained with fully-grown Braconid larvæ after vesicle has been withdrawn.

Experiments with later stage Braconids, which are preparing to leave the host and in which the vesicle has been withdrawn, showed, in all cases, an even body surface respiration.

Conclusions.

With regard to the caudate Ichneumonid larva it is safe to conclude from these experiments that the tail is completely negligible as a respiratory organ. This conclusion is fully in accordance with the fact that the tail diminishes in relative size as the larva grows, even though respiration is still entirely cutaneous.

The sponsors of the respiration theory have attempted to overcome this last objection with the statement that as soon as the tail disappears a tracheal system with open spiracles is developed. There are, however, serious flaws in this argument. Firstly, in every case known to the writer, the tail is definitely reduced in size before the spiracles are in contact with the air.

Secondly, although in some cases it may be true that the drying up of the host's body fluids results in the open spiracles gaining access to the air sooner than is supposed, yet there are many instances in which the tail is greatly reduced by the commencement of the second stage, whereas the larva is endoparasitic in a liquid medium till the end of the third instar.

To account for such inconvenient objections, many writers have assumed that an open spiracle system can function by diffusion of dissolved oxygen from the host's blood into the open spiracles even though the larva is totally immersed. Such a theory seems to the writer quite incredible, and experiments on such larvæ with cultures and indicators give it no support whatever. A small tracheal system exposed to the air will function solely by diffusion.

In air the rate of diffusion of a gas is so rapid that even the minute tracheal openings are sufficient. But when the gas is dissolved in a fluid its diffusion rate is, as is well known, very greatly retarded. That a simple tracheal system of a type suited to aerial respiration could also function efficiently when immersed in a liquid with a low O_2 tension such as insect blood, appears highly improbable, however low the oxygen tension within the tracheal system might be. Such a system can only work when there is a large gas surface in contact with the fluid to act as a diffusion membrane; as is the case in many adult aquatic insects which carry "air stores" entangled in the pubescence of the ventral surface, and thus in contact with the spiracular openings. But in the case of the parasitic larva the only surface of contact is the minute area in the mouth of each spiracle.

In those Braconids (*e.g.*, *Apanteles*) in which the vesicle is large and an efficient circulation is present, the experiments show that it is responsible for an appreciable proportion of the total respiration, but, while it undoubtedly serves to increase the respiratory surface, it is not a gill in the sense of being the sole or even the chief respiratory organ. Even where it attains its greatest development gaseous exchange is taking place at a rate little, if at all, faster than that at the greater part of the body surface.

In both *Microgaster* and *Apanteles* simple calculations indicate that the surface area of the vesicle at the time of its maximum development amounts to approximately 27 per cent. of the total area. We are probably safe in assuming from this that the vesicle, at the most, does not account for more than one-third of the total respiration. Nevertheless, it is quite probable that for a larva as large as *Apanteles popularis* (length 6 mm. to 8 mm.) such an increase in respiratory surface is of considerable value. In forms where the vesicle is small or the blood circulation inefficient (*e.g.*, *Orgilus*), its respiratory function is much less and may be almost nil.

The fact that the vesicle functions even to some extent as a gill indicates that the blood must act in some degree as a carrier of oxygen. There is very strong circumstantial evidence to show that this is the case in many insects (see Wigglesworth, 1931), but actual proof is lacking, save in those forms having hæmoglobin.

Perhaps the most striking example is that of the terrestrial larva of the Ceratopogonid *Forcipomyia* (Frew, 1923) which lacks open spiracles. Here there seems no doubt whatever that the blood must be respiratory in function. In the aquatic Chironomids, which have a closed and very poorly developed tracheal system, the work of Leitch (1916) has shown that even though hæmoglobin is present it is non-functional at pressures above 7 mm. of oxygen, and that under these conditions simple physical solution, quite apart from the presence of a pigment, is sufficient to supply all the needs of the insect. There are also many other Chironomids of similar structure and mode of life which lack hæmoglobin entirely.

There remains the question as to whether the vesicle has any other function besides that of respiration. Grandori, working with *A. glomeratus*, concludes from histological evidence that its chief function is that of excretion. He describes " excretory droplets " in the outer margin of the epithelial cells and also in the cuticular layer. These " droplets " can also be seen in *A. popularis*, but are less conspicuous in *Orgilus*. Whether they are truly excretory appears very doubtful. As Gatenby (1919) observed in *Microgaster* the outer part of the cells is crowded with "lipoid" matter in the form of a "granular cloud." Granules of this type are clearly visible in *A. popularis* ; in the fresh condition they stain with osmic acid but not markedly with Sudan III.

In addition to excretion and respiration, Grandori also assigns an absorptive function to the vesicle because he describes the fore gut as closed in the second instar. This closure does not appear to have been recorded in any other species and the stomodæum is definitely open in all those I have seen. In *A. popularis*, however, no solid food is absorbed till towards the end of the second instar; previous to this blood only is ingested, although *Orgilus* and *Microgaster* commence to take in solid matter at an earlier stage. As a rule the vesicle shows no signs of fat absorption till the later larval life, when in *A. popularis* and *Microgaster* a certain amount of true fat is undoubtedly present, but not as a rule before the fat body of the host is being taken into the gut. If, therefore, food absorption is one of the functions of the vesicle in the early stages it must be chiefly concerned with substances other than fat.

Summary.

(1) The object of the work described was to obtain some direct evidence as to the alleged respiratory function of the " tail " of endoparasitic Ichneumonid larvæ and the " caudal vesicle " of Braconids.

(2) Methods of biological indicators for studying the oxygen absorption are described in detail. The flagellate *Polytoma uvella* was the form found to be the most useful, since oxygen tension was shown to be the main factor controlling its movements. The luminous *B. phosphorescens* was also employed, the luminescence of which is dependent on the presence of oxygen. Carbon dioxide output was investigated mainly by means of p_H indicators.

(3) It is concluded that the "tail," which is such a characteristic feature of many first instar Ichneumonids, is of no importance for respiration. It is shown that the experimental results are in accord with those obtained from a study of structure.

(4) With regard to the caudal vesicle of the Braconids, it is shown that there are considerable differences of structure in different genera. Where the vesicle is large and supplied with a good blood circulation, experiments show it to be of undoubted importance as a respiratory organ. It is, however, not the sole, nor even the most important, organ of respiration, gas exchange taking place over the whole body surface. It is concluded that in *Apanteles* and *Microgaster* the vesicle, when at its maximum development, cannot be responsible for more than about one-third of the total respiration. In other forms, *e.g.*, *Orgilus*, its respiratory function is less.

(5) Other theories as to the function of the caudal vesicle are briefly discussed.

Acknowledgments.

The work here described was begun at the Entomological Department of the University of California, Citrus Experiment Station, at Riverside, California. Later, opportunity offered to continue it at the Farnham House Laboratory of the Imperial Institute of Entomology, where a great variety of material was easily obtainable during the course of parasitic investigations being carried on there. To Professor H. S. Smith and Dr. W. R. Thompson I am indebted for helpful interest in the work, and to my colleagues at Farnham House for assistance in obtaining certain species for experiment. I am indebted to Professor R. A. Peters, of Oxford, and to Miss Muriel Robertson and Dr. St. John Brooks, of the Lister Institute, for supplying me with the original cultures of Protozoa and Bacteria respectively and for advice as to the best methods for growing in pure culture. I also wish to thank Mr. J. T. Saunders for helpful advice on certain matters.

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The Development and Function of the Heart and Pericardium in Echinodermata.

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[PLATES 40–43.]

The investigations which form the subject of the paper were begun with the object of verifying the statements made by several authors with regard to the origin and development of the so-called "heart" or pericardial vesicle of Echinoderms. A study of the literature soon led to the conclusion that our knowledge of the development of the organ was somewhat defective and that a thorough revision of its development and ultimate fate would be desirable. This work has occupied my attention for the last two years and the results obtained have been fairly satisfactory.

Historical Resumé.

The Echinodermata offer a number of most interesting problems to the comparative physiologist. They are in many senses the lowest animals, from the point of view of organisation, which possess a true cœlom or secondary body-cavity. It was always assumed by earlier naturalists that these animals must have a circulatory system and strenuous efforts were made to find a heart and blood-vessels. None of these efforts has been very successful because the so-called vessels were found to be mere rents in the loose connective tissue without proper walls of their own, and further, no connection could be traced between vessels in one part and those in another part. The fact that these vessels owe the honour of being denominated blood-vessels at all is because they contain a ground-substance, which unlike the ground-substance of the rest of the connective tissue, stains with aniline dyes such as eosin and methyl green. If there is to be true circulation some part of the system must be rhythmically contractile and so a heart had to be found. A pillar-like organ, lying alongside the stone-canal in Echinoidea, Ophiuroidea and Asteroidea, was selected for the rôle. Unfortunately, in Holothuroidea, where the so-called vessels are best developed, it is absent and this fact may be correlated with the elongated shape of the animal and the contractility of the body wall. Later, as repeated observation had failed to detect any sign of its beating, the non-committal term of " pseudo-heart " was adopted for it. It is now proposed to call it the "pericardial vesicle."

As far back as 1863, Hensen (1863, p. 246), in his description of Brachiolariæ captured in Kiel Harbour, had mentioned that there was a pulsating heart-vesicle, on the dorsal side over the junction of the œsophagus and the stomach, which contracted about five or six times a minute. Later Metschnikoff (1869, p. 63) referred to the presence of a similar organ close to the pore-canal in late *Echinid plutei*. These observations seem to have been lost sight of, for the next reference is an independent observation of Bury's (1889, p. 74) who observed a slight pulsation of the organ in both Echinids and Asterids. He called it the "dorsal sac." Field (1892) discovered it in the Brachiolariæ of *Asterias vulgaris*, but he does not record any pulsations. Goto (1898, p. 55) is rather confused in his account of the pericardial vesicle as he seems to have mistaken it for the epigastric cœlom. Masterman (1902, p. 388) refers to this vesicle in *Cribrella* as the central cœlom and mentions that it goes to form the cardiac vesicle of the adult. MacBride (1896, p. 349; 1903, p. 299), working on *Asterina gibbosa* and later on *Echinus miliaris*, *E. esculentus* and *E. acutus*, identifies the pericardial vesicle with the right hydrocœlic rudiment. In his (1918, p. 40) paper on Echinocardium he rejects that view and refers to it as the madreporic vesicle. A good reference to the pericardial vesicle is found in the works of Gemmill (1912, 1914, 1915), who was able to identify it in *Solaster endeca*, *Asterias rubens* and *Porania pulvillus*. He was able to see pulsations of this vesicle in all the types mentioned above.

Materials and Methods.

The material for this work was obtained by artificial fertilisation of sea-urchins and star-fish at the Huxley Research Laboratory. The cultures were fed on Nitzschia.

The larvæ were preserved mostly in ½ per cent. osmic acid followed by Müller's fluid and then by gradual changes transferred to 90 per cent. alcohol. A few larvæ were fixed in Flemming's fluid without acetic acid and Bouin's fluid.

For purposes of section cutting the celloidin in paraffin method of embedding was used. The sections were cut mostly transversely as in such sections the pericardial vesicle could be studied clearly. They were stained usually in Delafield's hæmatoxylin which was sometimes followed by alcoholic eosin.

DESCRIPTION.

Echinus miliaris.

In serial sections of larvæ about 25 days old the pericardial vesicle appears as a small horizontally flattened vesicle on the dorsal aspect above the junction of the œsophagus and the stomach, immediately to the right of the pore-canal. Since it is situated dorsally to the main portion of the right cœlomic sac and is at the same time nearer to the median plane, it is impossible to show at any one particular section its true relations, and its development can only be studied in serial transverse sections.

Stage I. Origin and Early Development.—The pericardial vesicle makes its appearance about the seventeenth day. By this time the third pair of larval arms have begun to develop. On the left side the cœlomic sac has divided into a left anterior and a left posterior cœlom. The stone canal and the left hydrocœle are clearly marked out. The rudiment of the invagination which goes to form the ambulatory surface or "oral disc" has been formed. On the right side the cœlomic sac has divided into anterior and posterior halves. There is no sign of the formation of any structure corresponding to the hydrocœle on the right side. When serial transverse sections of larvæ about 17 days old are examined, and the right anterior cœlom traced backwards carefully, it is found to taper off into a narrow string of cells. This string extends almost to the mid-dorsal line of the œsophagus and there ends in a thickening (Plate 40, fig. 2). The growth of the thickening is inwards towards the mid-dorsal line, that is towards the left side of the right anterior cœlom. In other words, it extends dorsally and not posteriorly. Between the nineteenth and the twenty-first days after fertilisation the thickening becomes separated from the right anterior cœlom and forms a little solid mass of cells lying dorsal to the œsophagus to the right of the pore-canal (Plate 40, figs. 3 and 4). At this stage it was seen by Bury (1889) and Gemmill (1914). Sometimes this mass is seen to be connected with the right anterior cœlom exactly as MacBride (1903) saw it in *Echinus esculentus* and *E. miliaris*. This cellular knob may consist of three or four cells. The posterior part of the right anterior cœlom from which these cells originate is at a slightly higher level than that part of the left anterior cœlom from which the left hydrocœle takes its origin. In certain abnormal larvæ of *E. miliaris* a right water-vascular rudiment is developed exactly like that on the left side, below the point of origin of the madreporic vesicle. The growth of this right hydrocœle is posterior-wards.

Stage II. 21–30 *Days.*—At about the twenty-third day this solid nodule of cells acquires a lumen in its middle (Plate 41, fig. 5). The cavity thus formed extends and the cells multiply and are pushed outwards towards the periphery to form the walls of the vesicle. The pericardial vesicle is more or less rounded in shape and the cells bounding its walls are arranged rather closely and not unlike the cells bounding the water-pore. By this time the posterior divisions of the cœlomic sacs have extended backwards so as to meet each other at the posterior pole of the larva. The invagination which is to form the oral disc of the adult is now closed off from the exterior. Its cavity, the amniotic cavity, has come into close contact with the flattened pentagonal hydrocœle. The left posterior cœlom has sent forward a dorsal horn. Now, the vesicle gradually increases in size extending dorsally towards the middle line (Plate 41, fig. 6). When it has just become hollowed out it is definitely to the right of the middle line immediately to the left of the right anterior cœlom. The more or less circular shape is lost and it becomes horizontally flattened. At the same time the cells in its thin wall become flattened and elongated and draw apart from one another. The wall of the vesicle is thin and is composed of a single layer of loosely-set flattened cells. The vesicle begins to pulsate at about this time. The pulsations occur in the floor (ventral wall facing the œsophagus) which is periodically pushed upwards. The pulsations occur about 25 times a minute. When sections of larvæ about 30 days old are examined the pericardial vesicle is still a flattened thin-walled pulsating sac. No invagination of any kind is seen. In other words no trace of a heart as distinct from pericardium is distinguishable.

Stage III. 30 *Days to Metamorphosis.*—During this period the echinus rudiment grows considerably in size, the unpaired tube-feet acquire suckers, the rudiment of the tooth grows and assumes a "V" shape, and the first three pedicellariæ make their appearance. The first traces of the spines appear at about 33 days. Both right and left cœlomic sacs extend backwards to the anus. Between 35 and 40 days the echinus-rudiment becomes so large that it occupies the entire length of the larva. The hydrocœle becomes distended, and presses against the left side of the stomach so as to flatten it. The dental sacs and their contained teeth are greatly enlarged and send out diverticula which go to form the radial perihæmal canals. Later, traces of the dorsal spines and the adult œsophagus make their appearance.

In sections of larvæ at this stage, ranging from 30 to 45 days, the vesicle has undergone a change. In serial sections we are able to distinguish in the vesicle a lower invaginated portion and an outer covering layer (Plate 41, fig. 7). The invaginated portion is nothing but the in-pushed ventral wall or floor of the sac, and the enveloping layer is the dorsal wall or roof of the sac. What really happens in late plutei therefore is that the floor of the sac is invaginated into it and bears the same relation as the heart of a vertebrate does to its pericardium. We propose to call the invaginated part the "heart" and the covering layer the "pericardium." The extent to which this invagination is seen in sections varies in different specimens apparently depending upon the mode of preservation. The vesicle itself is more or less crescentic in shape and is widest in the middle, being narrower at the ends. It measures about 0·06 mm. from end to end and 0·04 mm. in the middle portion. It is situated nearly in the middle line and one end of it almost touches the axial sinus and the stone-canal (Plate 42, fig. 8). The heart portion is in open communication with the blastocœle below. In sections of larvæ preserved in osmic acid and Müller's fluid the heart portion stains more deeply than the rest of the blastocœlic ground-substance immediately surrounding the stomach. The fluid which fills the heart was not unlike the blastocœlic jelly and stray cells were occasionally seen in the heart portion though the pericardial portion remained unstained. The pulsations have now quickened considerably, ranging from 35 to 40 per minute.

Stage IV. After Metamorphosis.—Soon after metamorphosis in sections of the imago there appears on the aboral side of the water-pore a closed vesicle. The protrusion of its basal wall which can be compared with the heart of *Balanoglossus* is very prominent and the vesicle as a whole is rather crescentic in shape (Plate 42, fig. 9). A solid cord of cells growing into the jelly extends inwards towards the concavity of the vesicle which now is filled by the blastocœlic ground-substance. This closed vesicle is Prouho's "espacé madreporique " and the portion of the cord projecting into it is his " processus glandularis," the " dorsal organ " of Bury or the " ovoid gland " of MacBride. The position of this vesicle is exactly that of the pericardial vesicle of the pluteus, for what is on the right of the pore is on its aboral side in the adult. On the left side of the vesicle is the ampulla or the cœlomic enlargement into which the pore-canal opens and from which the stone-canal originated. The axial sinus also opens into it, but no connection of the vesicle with the axial sinus is apparent. Situated immediately under the madreporic plate the pericardial vesicle is distinct from the surrounding structures.

Asterias rubens.

Stage I. Origin and Early Development.—At the stage when the pericardial vesicle first appears in *A. rubens* the right and left anterior cœloms are prominent. They have not yet united in the pre-oral lobe of the bipinnaria. The right and left posterior cœloms have extended backwards to about the middle of the stomach. The pore-canal is very prominent. The vesicle arises in the same way as in *Echinus miliaris*. When transverse sections of larvæ about 17 to 18 days after fertilisation are examined a thickening appears on the right anterior cœlom above the junction of the œsophagus and the stomach (Plate 42, fig. 11). In the next stage this thickened portion is seen to be cut off from the anterior cœlom and lies towards the right side of the middle line and is nearer the right anterior cœlom (Plate 43, fig. 12). There is a strand of cells connecting this solid nodule with the right anterior cœlom.

In a slightly older larva, about 21 days old, there is a cavity being formed in the solid mass of cells, exactly in the position in which the pericardial vesicle appears in later larvæ, that is, more or less in the mid-dorsal line towards the right of the pore-canal. The stage in which the solid nodule acquires a cavity I was not able to obtain. But when the vesicle is just marked out there is a disconnected string of two or three cells proceeding from it towards the right anterior cœlom.

Stage II. 24 to 30 Days.—About the twenty-fourth day in serial sections the pericardial sac appears as a distinct vesicle. By this time the right and left anterior cœloms have united in the pre-oral lobe and the formation of the ventral horn of the left posterior cœlom has commenced. The vesicle is small and horizontally flattened. It is narrower at the right end and wider on the side facing the pore-canal (Plate 43, fig. 13). Its position is slightly lower than that of the corresponding structure in *E. miliaris*. The wall of the vesicle is thin, being composed of a single layer of horizontally flattened cells.

Pulsations occur now at the rate of 6 to 7 per minute. About the twenty-ninth day the left posterior cœlom and the right posterior cœlom are cut off from the respective anterior cœloms and the hydrocœle pouches become distinct. The vesicle does not change in appearance much; only it is longer and narrower extending to very near the pore-canal. There is no trace of any connection with the wall of the pore-canal. In sections no invagination of any kind is seen, but in living larvæ the floor of the sac is seen to be invaginated into it at intervals. The pulsations have increased slightly, being about 10 to 12 a minute.

Stage III. 30 Days to Metamorphosis.—About the thirty-fifth day after fertilisation the brachiolarian arms and the fixation disc appear. Soon the ends of the brachiolarian arms become papillated and two papillæ appear on either side of the fixation disc. A few days later the larva fixes itself by means of the fixation disc and metamorphosis into the imago begins. During this time the pericardial vesicle increases in size and as the adjacent cœlomic cavities have also been expanding it comes to be very close to the middle portion of the right anterior cœlom, the dorsal angles of the left posterior cœlom, and the right posterior cœlom and, lastly, with the middle part of the left anterior cœlom (Plate 43, fig. 14). From all these cavities it remains distinct during larval life as well as during metamorphosis. In later brachiolariæ immediately preceding fixation the vesicle measures 0·2 mm. in length and 0·1 mm. in breadth, and thus bears no inconsiderable proportion to the size of the larva. Meanwhile the basal skeletal plates have appeared and the largest of these overlies the pericardial vesicle rendering the observations of the pulsations difficult. At about this time the heart beat ranges between 10 and 12 per minute. In sections, the floor of the vesicle appears slightly thicker than the roof, but this is probably due to the presence of muscular fibres which are necessary for the contractions. In the late larval stages just before metamorphosis the floor of the vesicle is raised upwards owing to the invagination of the vesicle by the underlying spongy tissue to form the heart (Plate 43, fig. 15), which resembles the similar structure which we found in *E. miliaris* and which we regard as homologous with the pericardial vesicle and heart of *Balanoglossus*.

Stage IV. After Metamorphosis.—In sections of the young star-fish the pericardial vesicle appears as a closed sac immediately under the calcareous madreporite. Its floor is now permanently invaginated by the spongy tissue underneath, which projects into the concavity as the so-called head-process of the axial organ. To the left of the sac are the stone-canal, the axial sinus and the madreporic pore. Over the pericardial vesicle aborally is a diverticulum from the stone-canal funnel, this is the ampulla of the stone-canal. A special portion of the genital stolon projects into the heart.

Pulsation.

The point of greatest interest noted in observations from day to day on the living larvæ of *E. miliaris* and *A. rubens* was that the wall of the sac, particularly its floor, contracts in a rhythmic or sub-rhythmic manner. The larvæ form most suitable objects for the study of the pericardial vesicle because of their extreme transparency. Placed under suitable illumination and in a proper position, rhythmical contractions can be clearly seen, and I have observed them both from a lateral and a dorsal aspect. When I first observed the contractions they seemed to occur at considerable intervals, about 1 or 2 a minute. Then it occurred to me that the larvæ were probably suffering from asphyxiation and as I had a culture with a considerable number of larvæ swimming about in it, I placed this beside the microscope, removed larvæ from it, placed them in sea water in a hollow slide and examined them immediately under the microscope. I found that under these conditions the vesicle in both echinoplutei and bipinnariæ made much more rapid pulsations. The dorsal aspect of the larvæ is most suitable for studying the pulsations in star-fish and the lateral in the sea-urchin, as on the dorsal side the vesicle is sometimes overlapped in the latter by clusters of pigment spots and can only be studied with great difficulty. In recording the number of beats per minute the average number of beats of a large number of larvæ, examined at any one particular stage, was taken into consideration.

The contractions of the vesicle were first recognisable in *Echinus* about the twenty-first day and about the twenty-third day in the case of *Asterias*. A specimen of *Echinus*, in which the so-called rudiment had attained only half its ultimate size, had a vesicle beating about 25 times a minute. One which was full grown and just about to metamorphose and in which the larval œsophagus was already occluded showed a vesicle with 40 pulsations a minute. There was, therefore, a definite increase in the number of pulsations with the growth of the larvæ.

In *Asterias*, about the twenty-fifth day after fertilisation, contractions occurred once in every 7 or 8 seconds. Later there was not an appreciable increase in the rate of beating. During the fifth week there were about 10 or 12 contractions per minute, and the number of pulsations just before metamorphosis was in the neighbourhood of 12. The fact that the larval heart of the sea-urchin pulsates about four times as much as that of the star-fish is very significant. It is quite probable that this phenomenon is correlated with the activity and nourishment of the two animals in question.

The pulsations take place as already mentioned in the ventral wall of the sac. So far as one can judge, at intervals the floor of the sac swells irregularly upwards and is invaginated into the sac, being filled with a fluid from the underlying tissue spaces, and after the contained fluid is displaced the floor of the sac descends. The pulsations therefore begin at the posterior end and travel forwards exactly like the peristalsis of the intestine. The floor seems to move forwards with one or two pauses. This is clearly seen in the case of *A. rubens*. It moves almost half-way upwards and then there is a sort of pause, but this is of such short duration that it is very difficult to observe it except under the high power of the microscope with a coverslip on the larva in a drop of sea-water, on a hollowed glass-slide. Then with a jerk, gathering more fluid from the bottom, it moves upwards until it almost touches the dorsal wall of the sac and empties out its contents and then bulges downwards. The effect, therefore, of this irregular motion is to drive the contained fluid forwards. The fluid contained in the tissue spaces below is the blastocœlic fluid together with the juice exuded by the stomach cells as I have tried to prove in the chapter on "Discussion." The heart then pumps this fluid forwards towards tissues or organs removed further away from any other supply of nutritive juice.

The question now arises whether the heart goes on contracting in the adult life of the sea-urchin and star-fish. As mentioned before, the pericardial sac persists in the adult lying under the calcareous madreporite. Gemmil (1918) saw pulsations in young ophiurids, and I have been able to see faint pulsations in the young imago of *Ophiocoma*. Further, I have made out pulsations in both star-fish and sea-urchins in all stages of larval development as well as in early metamorphosis until the growing opacity of the body wall rendered further observations impossible. It seems reasonable to assume, therefore, that the heart goes on pulsating in the adult life of the sea-urchin and star-fish supplying nourishment to the required parts.

Effect of Magnesium Chloride and Potassium Chloride on the Rate of Pulsation.

The invaginated floors of the pericardial vesicle of Echinoderm larvæ are comparable to the hearts of other animals like *Balanoglossus* and as such they might be expected to be senstitive to the ionic composition of the fluid with which they are in contact. Mines (1912) found that the frequency of the heart beat of *Pecten* was reduced by the addition of magnesium to the perfusion fluid, whereas the addition of potassium ions tended to increase the frequency of beat. In the case of the pericardial vesicles of the Echinoderms here being considered it is not possible to effect true perfusion as with a heart that can be isolated, but it is of some interest to note that when the whole larvæ are immersed in fluids containing either an excess of magnesium or an excess of potassium ions, the frequency of beat of the floor of the vesicles changes in a way which is analogous to that of a perfused heart. The effect of magnesium was best observed by using a mixture of one part by volume of 0·4 molar magnesium chloride solution (containing a little sodium bicarbonate) with three parts of normal sea-water; the effect of potassium was observed in two parts of 0·6 molar potassium chloride solution (also containing a trace of sodium bicarbonate) with ten parts by volume of sea-water. The data in the table are typical of the results obtained.

Table.

Solution surrounding the larvæ.	Duration of immersion.	Average rate of beat in pulsations per minute (for 3 larvæ).	
		Echinus miliaris.	*Asterias.*
SERIES I.			
Normal sea-water ..	—	35	10
$MgCl_2$ sol	1	25	7
	2	24	6
	4	20	4
	8	13	1
	10	11	1
	12	9	—
Transfer to normal sea-water	1	24	4
	2	26	5
	4	29	6
	8	32	9
	10	34	9
	12	34	—
SERIES II.			
Normal sea-water	—	35	10
KCl sol	1	38	11
	2	40	13
	4	43	14
	6	44	15
Transfer to normal sea-water	1	40	11
	2	38	10
	4	37	9
	6	36	8

DISCUSSION.

Origin.

There is a good deal of confusion with regard to the origin of the pericardial vesicle in Echinoderms. Data regarding the origin of the vesicle in Echinoderm larvæ are provided by Bury, Field, Goto, Masterman, MacBride and Gemmill.

Bury (1889, p. 74) and Field (1896, p. 117) agree that the pericardial vesicle is of mesenchymatous origin. The former believes that it arises at a fairly early stage from a group of cells situated in the middle line over the œsophagus to the right rather than in front of the hydropore, and that it is certainly distinct from the cœlom in its origin.

Goto (1898, p. 255) concluded that the vesicle arose by an extension of the left anterior cœlom on the dorsal side of the stomach, and that this extension in the adult is incorporated in the axial sinus while the definitive pericardial vesicle is constricted off from the axial sinus at a later stage. Goto's account is, however, unsatisfactory, as he seems to have mistaken the pericardial vesicle for the epigastric cœlom in Plate XXI, figs. 19 and 20.

MacBride (1896, 1903, and 1918) in *Asterina* and other Echinoderms, Masterman (1902) in *Cribrella*, and Gemmill (1912) in *Solaster*, believe that the pericardial vesicle arises from the anterior cœlom, in the middle line according to Masterman, and a little to the right of the middle line according to the two others. In view of the fact that the single anterior cœlom both in *Cribella* and *Asterina* represents the result of the fusion of the anterior cœloms of different size, the difference between MacBride and Masterman is trivial. But, the pericardial vesicle is definitely to the right of the middle line in its early stages though it extends right over the middle line later on, as I have shown in the chapter on " Description."

Gemmill, on a subsequent examination of the larva of *Solaster endeca* and *Solaster papposus*, came to the extraordinary view that in both these species the pericardial vesicle arose as an ectodermal invagination. This statement about the mode of origin in the two species of *Solaster* is so contradictory and opposed to the results of the other workers that before taking it into consideration it would be desirable to await its confirmation. In his papers on *Asterias rubens* and *Porania pulvillus* Gemmill (1914, 1915) puts forward the view that the vesicle arises from mesenchymal cells budded off from the archenteron as early as the period at which the general mesenchyme is formed ; but, in reality, the pericardial vesicle grows out from the dorsal surface of the right anterior cœlom as a solid nodule of cells connected in the beginning with the cœlomic sac by a slender string of protoplasm. This connection is cut off and the solid nodule is isolated.

Gemmill must have identified these cells as those going to form the pericardial vesicle at this time and must have regarded them as mesenchymal for it is difficult to distinguish them from the latter.

Right Hydrocœle and Heart.

The origin of the pericardial vesicle from the dorsal surface of the right anterior cœlom must have led MacBride (1896, 1903 and 1907) to consider it as the right hydrocœlic rudiment. This view seems to have been put forward on comparative grounds to obtain exact comparisons between the structures of right and left larval origin and seemed to be corroborated by the fact that no pericardial vesicle could be detected in his double hydrocœled larvæ of *Asterina*. However, in his (1918) paper on *Echinocardium* he discarded that view as untenable since larvæ of *Asterias rubens* in which both a pericardial vesicle and two hydrocœles were present turned up in Gemmill's (1914) cultures, and similar larvæ were found by MacBride in *Echinus miliaris* and *Echinocardium*. Double hydrocœle larvæ of *Asterias rubens* and *Echinus miliaris* possessing a pericardial vesicle turned up in my cultures. The pericardial vesicle, therefore, arises from the posterior part of the right anterior cœlomic sac at a higher level than that part of the anterior cœlom from which the hydrocœle is given off.

The "Blood."

The floor of the pericardial vesicle or the heart is in open communication with the blastocœle below. In sections of larvæ preserved in osmic and Müller the heart stains more deeply than the surrounding blastocœle. This staining peculiarity is shared by the blastocœlic ground-substance immediately surrounding the stomach, and we may justly come to the conclusion that this difference in staining capacity is due to the exudation of digested material from the stomach cells. If this view is accepted the fluid or "blood" which the heart pumps forward is the blastocœlic fluid which contains the highly nutritious juice exuded by the stomach cells.

Probable Function.

I have not the slightest doubt that the pulsations of the pericardial vesicle have a physiological meaning. The contractions of the floor of the vesicle and the character of the spongy tissue underneath strongly suggest that we have here something of the nature of a hæmal node. A stream of nutritive material is exuded by the stomach where absorption is taking place and is sucked up by the heart as it contracts and is pumped forwards through its thin wall towards the surrounding organs which are far removed from any other supply of nutritive fluid than that provided by the cœlomic fluid. It is quite probable that most of this cœlomic fluid is taken up by the growing regions of the larva such as the *Echinus rudiment*, the water vascular rudiment, and that the anterior regions like the larval brain of *Echinus* and the mouth region of *Asterias* being bereft of enough nutritive fluid, are supplied with the required amount of nutrition by the contractions of the pericardial vesicle.

I believe that the pericardial vesicle goes on pulsating in the adult star-fish and sea-urchin, since contractions have been observed in young ophiurids, by Gemmill (1918).

Now, in the sea-urchin at metamorphosis, a portion of the ovoid gland projects into the cavity formed by the invagination of the floor of the pericardial vesicle which lies along the aboral portion of the axial sinus. At its aboral end the vesicle is close to a band of membrane which connects the intestine with the body-wall and within this band there is a tract of loose connective tissue with stainable ground-substance. It is quite a tenable view, therefore, that throughout adult life the pericardial vesicle continues its pulsations, pumping juices exuded by the stomach towards the body-wall. But at this point is found, according to MacBride, the infolding of peritoneal epithelium which gives rise to the genital stolon, which is the central point of origin of genital cells. Now, the genital cells in the sea-urchin as well as star-fish undergo annually an enormous growth. The difference in size, between the genital cells in the autumn and winter after genital products have been shed and in spring when the breeding season is coming on, is colossal. Hamann (1885) believes that it is extremely probable that the genital cells formed in the stolon wander away along the circular rod of genital cells called the genital-rachis to the positions in the interradii where the genital organs are situated. There must be periodically an enormous increase in the number of genital cells, for which an abundant supply of nourishment must be required, so we may quite reasonably put forward the view that the pulsations of the pericardial vesicle direct a stream of nourishment towards the growing genital cells.

In star-fish at metamorphosis the spongy tissue underneath invades the floor of the sac to form the so-called head-process of the axial organ with which it is in communication at its upper aboral end. The madreporic or pericardial vesicle is, as mentioned before, situated directly under the madreporite. The gastric hæmal tufts and the axial organ which are found near the vesicle, have been found to exhibit slow contractions by Gemmill (1915) and the so-called head-process by Prouho (1887) in *Dorocidaris*. The ampulla of the stone-canal which lies close to the vesicle may readily increase or decrease in size as it communicates with the axial sinus as well as the stone-canal. Such changes in volume might facilitate the pulsations of the pericardial vesicle and draw up hæmal fluid into the spongy spaces in its floor with greater ease. Knowing these facts we cannot but conclude that there is a sort of circulation of a nutritive fluid going on. Now, Ludwig's gastric hæmal strands have been found to be really hæmal in character by Gemmill (1914, p. 272); what probably happens, therefore, is that a fluid nutritive in function is transferred by the spongy channels of the gastric hæmal strands to the junction of the so-called head-process or heart with the axial organ. The genital rachis is an outgrowth of the axial organ and the genital organs are proliferations from the genital rachis. Surrounding the genital rachis is connective tissue containing "blood." This blood is fed from the blood pumped by the heart and it serves to nourish the growing genital organs. These are of far greater relative dimensions in *Echinus* than in *Asterias* and this difference probably accounts for the difference in the beating of the heart of the two forms.

Homology.

Now the most interesting circumstance about this heart as Bury pointed out, and as endorsed by Gemmill, is that a similar organ exists both in the larval and the adult stages of *Balanoglossus*. We owe to Metschnikoff (1869) the groundwork of the comparison between *Asterids* and *Balanoglossus*. Morgan (1891, p. 442) followed by Bury (1896, p. 128) and afterwards Masterman (1902) extended it to the important matter of the pericardial vesicle. Later, Gemmill (1914, p. 277) continued the comparison still further in the matter of the body and head of the axial organ, the principal hæmal trunks, the pharyngeal cœlom and the regions of the axial sinus. He homologised the pericardial vesicle with the pericardial cœlom, the head-process of the axial organ with the heart, the gastric hæmal tufts with the dorsal vessel of trunk, axial organ and oral hæmal ring with the left pharyngeal vessel and ventral vessel of trunk respectively. The heart is the protrusion of the ventral wall of the pericardial sac: it is situated above the mouth. In the larvæ of *Balanoglossus* as of Echinoderms there is an œsophagus which arises as an inpushing of the ectoderm. This œsophagus at metamorphosis flattens out and disappears and the position of the adult mouth corresponds to the inner end of the larval œsophagus. The heart in *Balanoglossus* drives the contained blood forwards towards the so-called excretory organ formed from the posterior wall of the proboscis cœlom. The blood is contained in vessels, but these vessels, as the study of their development shows, are nothing but the remnants of the larval blastocœle. Bateson (1884) describes the pericardial sac of Tornaria larva as arising as a solid bud from the posterior wall of the proboscis cœlom. The pericardial vesicle of Echinoderms arises in a similar way from the posterior part of the right anterior cœlom and drives the contained fluid forwards. In origin and structure then, the pericaridal vesicle of Echinoderms corresponds to the pericardial sac of *Balanoglossus* and the invaginated floor of the vesicle, with the inpushed spongy tissue, is comparable to the central sinus or heart. Therefore, it seems to me that it is quite justifiable to say that the structure and relationship of this organ constitute one of the best proofs of the affinity of Echinodermata with Enteropneusta.

Summary.

(1) The pericardial vesicle arises as a solid bud from the posterior part of the right anterior cœlom.

(2) It becomes hollowed out and extends dorsally.

(3) Pulsations occur in the floor of the vesicle and travel forwards. The heart of *E. miliaris* beats about four times as much as that of *A. rubens*.

(4) The fluid contained in the heart is the blastocœlic fluid with the rich juice exuded by the stomach cells.

(5) Magnesium chloride in small concentrations decreases the number of beats per minute : its removal causes the beats to quicken.

(6) Potassium chloride in small quantities produces an increase in the rate of contractions : its removal results in a slowing of the beat.

(7) In late plutei the floor becomes permanently invaginated in the vesicle forming the heart.

(8) In late brachiolariæ the spongy tissue underneath invades the invaginated floor of the vesicle.

(9) The vesicle persists in the adult star-fish and sea-urchin lying on the dorsal side directly under the madreporite as a closed sac.

(10) The pericardial vesicle of Echinoderms is homologous with the pericardial vesicle of *Balanoglossus*.

I take this opportunity to express my deep gratitude to Professor MacBride not only for the suggestion of *Asterias rubens* and *Echinus miliaris* as proper types to investigate, but also for much encouragement and advice in the course of this research. I must also thank Mr. C. C. John for his invaluable help in the preparation of the figures.

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LIST OF ABBREVIATIONS.

ax-sin., axial sinus. *e. rud.*, echinus rudiment. *gen. st.*, genital stolon. *ht.*, heart. *hyp. c.*, hypogastric cœlom. *int.*, intestine. *l.a.c.*, left anterior cœlomic sac. *l. hyd.*, left hydrocœl. *l.p.c.*, left posterior cœlomic sac. *m.p.*, madreporic pore. *n. rad.*, radial nerve cord. *oes.*, œsophagus. *oes. ad.*, adult œsophagus. *p.c.*, pore canal. *p.v.*, pericardial vesicle. *r.a.c.*, right anterior cœlomic cavity. *r.p.c.*, right posterior cœlomic sac. *rect.*, rectum. *st.*, stomach. *st. c.*, stone-canal.

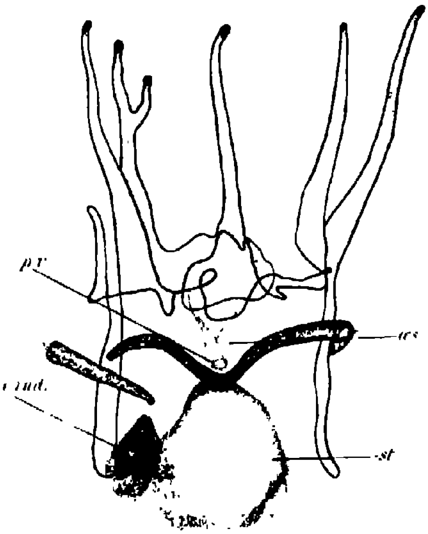


FIG. 1.

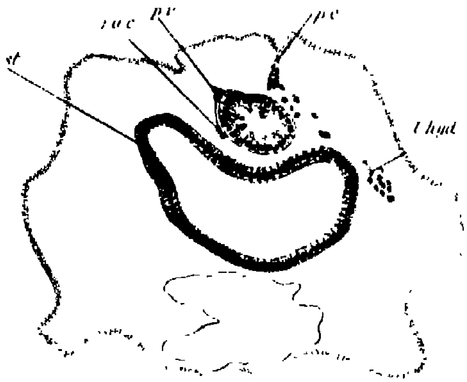


FIG. 2.

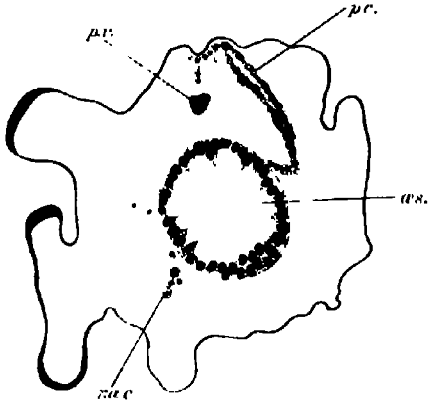
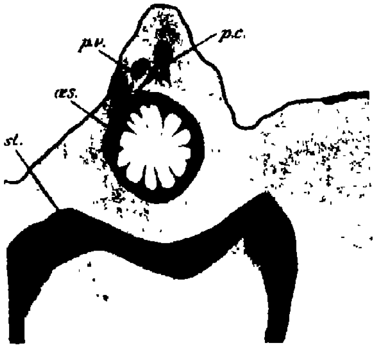


FIG. 3.



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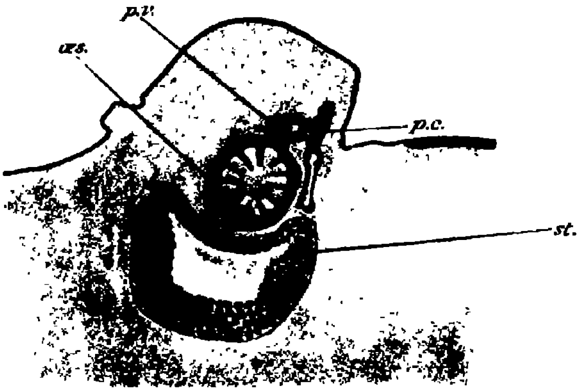

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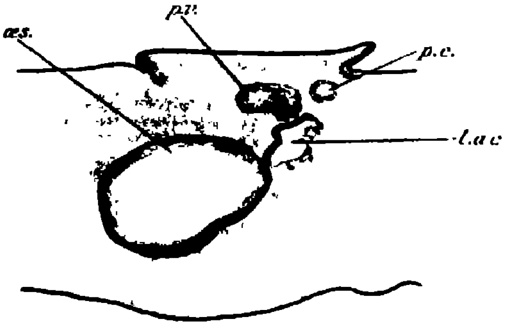

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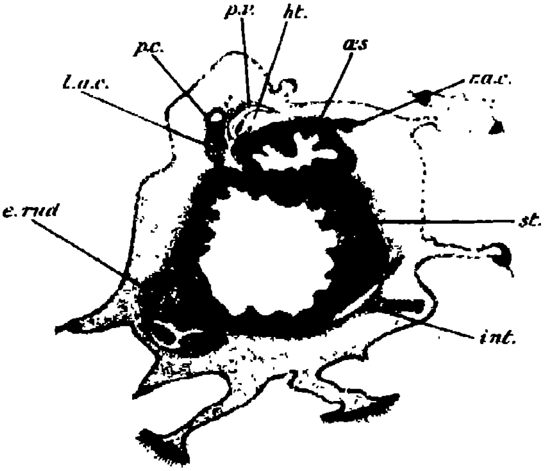

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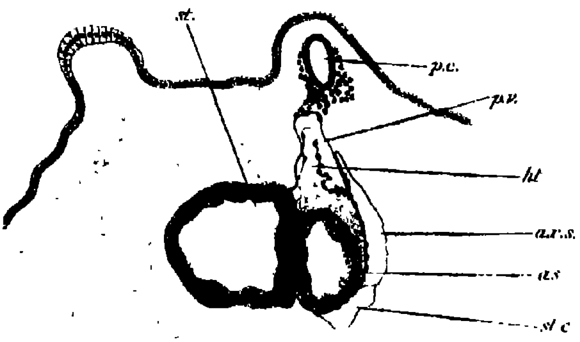

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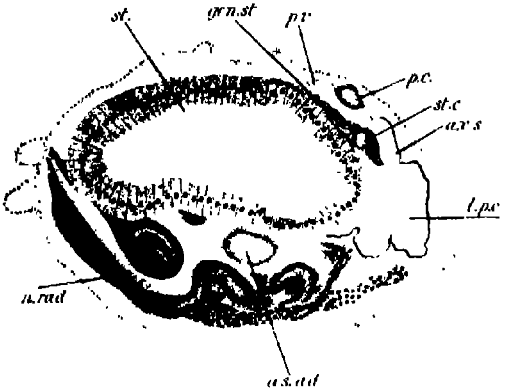

Fig. 9.

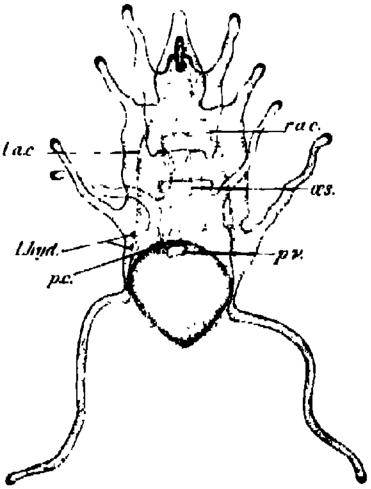

Fig. 10.

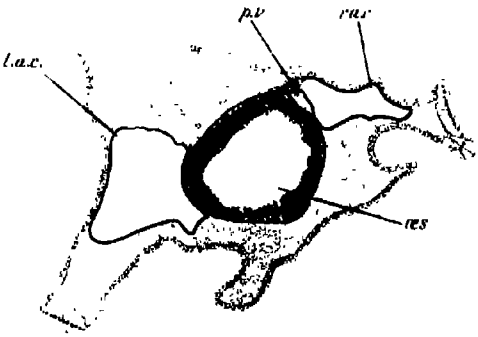

Fig. 11.

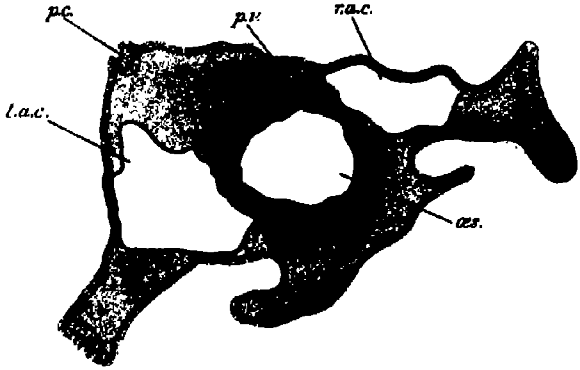

Fig. 12.

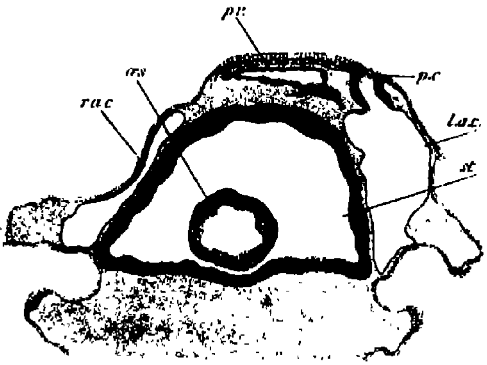

Fig. 13.

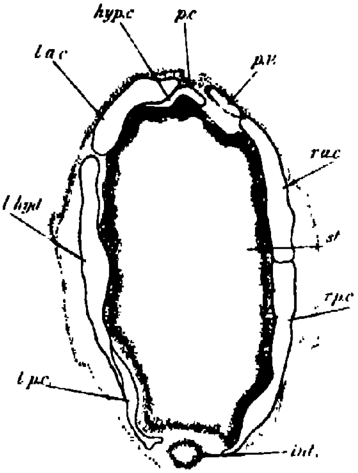

Fig. 14.

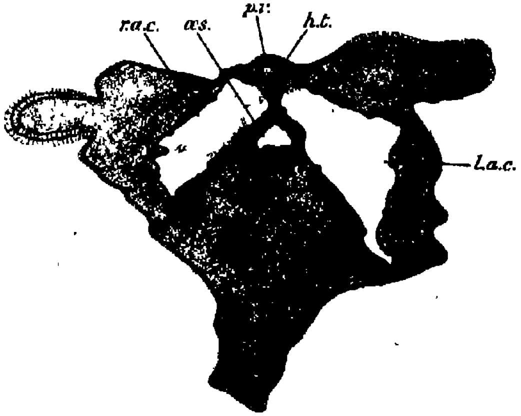

Fig. 15.

EXPLANATION OF PLATES AND FIGURES.

FIG. 1.—Drawing from a living larva of *Echinus miliaris* about 30 days after fertilisation, showing the position of the pericardial vesicle.

FIG. 2.—Transverse section of *E. miliaris*, 17 days old. The thickening of the right anterior cœlomic sac is clearly seen.

FIG. 3.—Transverse section of a larva slightly older than that in fig. 2. The thickening which is the rudiment of the pericardial vesicle is completely cut off from the right anterior cœlom.

FIG. 4.—Transverse section of larva of *E. miliaris*, 20 days old. The pericardial vesicle is in the form of a solid nodule lying to the right of the pore-canal.

FIG. 5.—Transverse section of *E. miliaris* about 24 days old. The solid nodule of cells is seen to have acquired a cavity and is rounded in shape.

FIG. 6.—Transverse section of larva of *E. miliaris* 30 days old. The pericardial vesicle is seen to be extending dorsally and horizontally.

FIG. 7.—Transverse section of larva of *E. miliaris* 30 days old. Pericardium and heart are distinctly seen.

FIG. 8.—Transverse section of larva of *E. miliaris*, about 40 days after fertilisation. The crescentic-shaped vesicle, the pore, the axial sinus and the stone-canal are distinguishable.

FIG. 9.—Transverse section through an imago of *E. miliaris* showing the pericardium and adjacent organs.

FIG. 10.—Larva of *Asterias rubens* viewed from the dorsal aspect. The pore and the pericardial vesicle are seen.

FIG. 11.—Transverse section of *A. rubens*, 18 days old (obliquely cut). The right anterior cœlomic sac is giving off dorsally a nodule of cells which is the beginning of the pericardial vesicle.

FIG. 12.·Transverse section (obliquely cut) of larva of *A. rubens*, about 18 days old. The rudiment of the pericardial vesicle is completely separated from the right anterior cœlom.

FIG. 13.—Transverse section of a specimen older than that shown in fig. 12. The pericardial vesicle is fully formed. The opening of the pore-canal into the left anterior cœlom is seen.

FIG. 14.—Longitudinal section of a larva of *A. rubens* about 35 days old. The anterior and posterior cœloms and the pericardial vesicle are distinguishable.

FIG. 15.—Transverse section of an older specimen of *A. rubens* immediately before fixation. The hydrocœle is well developed. The invagination of the spongy tissue into the floor of the pericardial vesicle is apparent.

577 . 16D + 547 . 926 . 2

Crystalline Vitamin D.

By F. A. Askew, R. B. Bourdillon, H. M. Bruce, R. K. Callow, J. St. L. Philpot, and T. A. Webster.

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(From the National Institute for Medical Research, London, N.W.3.)

In two earlier papers in these 'Proceedings' (Askew *et al.*, 1930, *a*; Angus *et al.*, 1931) a crystalline antirachitic substance was described, obtained by vacuum distillation of the products of irradiation of ergosterol. In the second paper, the physical properties and antirachitic activities of a number of successive preparations of this product were recorded, and it was shown that these were sufficiently constant to suggest that it consisted mainly of one substance. We avoided the conclusion that it was yet pure, the properties showing distinct variations which suggested that other, and presumably inactive, substances were present to a variable extent. For convenience, and with reference to its conspicuous biochemical activity, we provisionally applied the name "calciferol" to this crystalline product of distillation. We realised, however, that the substance to which that name could be properly and definitely applied, as being the pure antirachitic vitamin, might differ to some extent from the product then described. Among other indications in that direction was the fact that the biological activity of this product was not greatly in excess of that shown by many samples of the crude irradiation product. It was indeed suggested that the irradiation mixture might contain more than one active substance (*cp.* Bills *et al.*, 1931).

Since that second publication, progress has been rapid in more than one laboratory. A few weeks later Windaus (1931) gave, at a discussion meeting of the Royal Society, a preliminary description of a crystalline product which he had separated after partial condensation of the mixed products of irradiation with maleic or citraconic anhydride (Diels' reaction). Its melting-point, absorption and antirachitic activity were practically identical with those of our product, but its optical dextrorotation was so much lower that the two preparations could not be identical.

Shortly after this, we obtained, with slight variations of the distillation procedure, other samples of our crystalline product with even higher dextro-rotations, but still the same biological activity. We then found that deliberate heating of the product caused a further large rise of optical rotation, with loss of antirachitic activity. Hence it seemed highly probable that our original preparations of "calciferol" contained a product of thermal transformation in addition to the active principle. These constituents were quite inseparable by fractional crystallisation of the product itself, but we found, in the 3 : 5-dinitrobenzoates, derivatives so differing in colour and crystalline form that a separation was easily effected. Two sterols so obtained were mentioned by one of us (Callow, 1931) at a discussion in the Chemistry Section of the British Association for the Advancement of Science on September 25. One of them was identical with the substance produced by heating, and was referred to as "pyrocalciferol"; the other was the calciferol with which this paper is mainly concerned. This was found, in biological tests completed shortly afterwards, to have an antirachitic activity about twice as great as that of the preparation from which it was separated, or of Windaus's first crystalline product (vitamin D_1). Meanwhile, a further publication was made by Windaus and his colleagues (Windaus *et al.*, 1931, *a*) in which the crystalline product already described was further characterised, and was now termed vitamin D_1. In an addendum (dated September 19) to this paper, there was mentioned for the first time the isolation by Linsert of a second crystalline substance, which was referred to as vitamin D_2. The optical rotation and melting-point of vitamin D_2, given in this addendum, were similar to those of the active substance which we had recently isolated through the dinitrobenzoate. The identity of the two substances could not, however, be assumed on the basis of these facts alone, particularly since Windaus and his co-workers had not recognised the fact that vitamin D_2 possessed about twice the antirachitic activity of vitamin D_1, but stated that it was "at least as active" as vitamin D_1, and regarded the two as independent antirachitic principles.

At this stage we published a preliminary account of our later results (Askew *et al.*, 1931) which included the preparation of calciferyl 3 : 5-dinitrobenzoate directly from the crude product of irradiation without preliminary distillation. In a paper subsequently published (Windaus *et al.*, 1931, *c*), Windaus assumed the existence of two vitamins, D_1 and D_2, not greatly dissimilar in activity, but modified this view in an addendum, in which they identified their vitamin D_2 with our pure calciferol, and stated that, by using the method of separation as dinitrobenzoates, vitamin D_1 could be resolved into vitamin D_2 and an isomeric alcohol. We reached independently the same conclusions as the result of work described in detail in this paper, and based partly on the examination of samples of vitamin D_1 and vitamin D_2 which Professor Windaus most kindly sent to us when we suggested an exchange of specimens.

Thus the two sets of investigations, although carried out by different methods until the final stage, have followed courses in some respects similar. Our original impure preparation of calciferol has been found, in fact, to contain only 50 per cent. of calciferol itself, and to be a solid solution of molecular compounds formed by calciferol with two different biologically inactive sterols. One of these is the substance called "pyrocalciferol," the other a substance which we had known as "X," and which proves to be identical with the inactive component of Windaus's vitamin D_1. Vitamin D_1 appears to be a simple molecular compound of calciferol (vitamin D_2) and this inactive substance, which until it receives a more permanent name we shall call sterol X.*

In these circumstances a word should be said on the subject of nomenclature. When, in our previous paper, we provisionally applied the name "calciferol" to the crystalline product obtained by distillation, we used it with reference to an antirachitic principle, which we believed we had obtained with a small admixture of inert substances. Now that it is clear that the active principle constituted only one-half of the original product, the name might be regarded as not strictly applicable to the more active product now isolated. However, the term vitamin D is inconvenient when referring to esters and other derivatives of the compound, and the adoption of a new name at this stage would tend to cause confusion. We therefore use the term calciferol for the purified substance throughout this paper. We are, however, not unmindful of its independent isolation by Linsert, and we use the name without claiming the exclusive right to determine the nomenclature of this compound.

Preparation of Calciferol.

Calciferol was first obtained in this laboratory by treating with 3 : 5-dinitrobenzoyl chloride the "calciferol" prepared by distillation, and subsequently hydrolysing the ester, after separation by fractional crystallisation. However, an identical product can be prepared more simply by the following procedure, which omits the distillation.

Irradiation.—Ergosterol was irradiated in an ethereal solution, while flowing through a water-cooled silica spiral round a mercury lamp, as described previously, but with the insertion of a 1 per cent. solution of xylene in alcohol between lamp and spiral, to exclude wave-lengths shorter than 275 mμ. The rate of flow was adjusted to give a transformation of 50 to 70 per cent. of the ergosterol used. After evaporation of the ether, the irradiation products were extracted with cold 90 per cent. alcohol. The solution was freed from residual ergosterol by precipitation with digitonin, followed by dilution with water, and extraction with light petroleum. The extract was evaporated to dryness, and the resultant resin dried over phosphorus pentoxide *in vacuo*, and esterified as in the following example.

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* While this paper was in the press, a paper was published by Lüttringhaus (1931, 'Chem. Z.,' vol. 55, p. 956) describing this compound as "Lumisterin." We assume that this will be taken as the permanent name for this substance.

Esterification.—5·5 g. of resin of $[\alpha]^{20}_{5461}$ + 15° (in alcohol) was dissolved in 10 c.c. of dry pyridine and the solution added to a mixture of 5·5 g. of 3:5-dinitrobenzoyl chloride and 15 c.c. of dry pyridine. The contents of the flask were well stirred, and heated in a boiling water-bath for 15 minutes. After cooling, water was added, the mixture was stirred, and the water poured off from the dark oil. The latter was then repeatedly stirred with fresh quantities of water until practically free from pyridine; it became extremely viscous, and lighter in colour. The adherent water was finally shaken out of the flask, and the material boiled with 10 c.c. of 97 per cent. alcohol, cooled, and the alcoholic solution poured off and rejected. The residue was then warmed and stirred with 10 c.c. of acetone. Crude calciferyl 3:5-dinitrobenzoate separated rapidly, and was collected, washed, and dried; weight 3·0 g., melting-point 138° to 142°, $[\alpha]^{20}_{5461}$ + 70° in benzene. A second crop, 0·6 g., melting-point 138° to 141·5°, separated from the mother-liquors after slight evaporation and keeping at + 4°. The first crop was recrystallised three times from acetone, once from a mixture of benzene and alcohol, and then once again from acetone, giving 1·8 g. of the final product, as lemon-coloured crystals of melting-point 147° to 149° and $[\alpha]^{20}_{5461}$ + 69° in benzene.

Hydrolysis.—1·7 g. of the above dinitrobenzoate was finely powdered and suspended in 20 c.c. of boiling alcohol. 2 c.c. of 2N aqueous sodium hydroxide solution was then added drop by drop, and after 15 minutes the ester dissolved completely. The solution was then filtered and diluted with water to 50 c.c. The oily precipitate which formed crystallised on cooling and scratching the side of the flask. The solid, when collected and dried, weighed 1·14 g. On recrystallisation from 7 c.c. of methyl alcohol, 0·7 g. of calciferol was obtained, showing the physical constants given below.

Variations in Method of Preparation of Calciferol.

(*a*) *Use of Radiation of Different Wave-lengths.*—Some tests were made to determine the effect of using radiation of different wave-lengths in the irradiation of ergosterol, on the yield of calciferol prepared as above. With unfiltered light from a mercury vapour lamp, the crude calciferyl 3 : 5-dinitrobenzoate which separated corresponded to a yield of from 14 to 33 per cent. of calciferol from the ergosterol transformed. In three preparations made with light filtered through xylene solution, to exclude radiations of wave-length shorter than 275 mμ, the yield varied from 38 to 44 per cent., and in one experiment which was made with the "short wave" lamp described by Drs. Reerink and van Wijk (1929), and very kindly provided by them, the yield obtained was only 10 per cent. It is believed that these differences correspond to variations in the antirachitic activity of the crude resins, although our biological data are insufficient to make this certain. These results are consistent with the observations of other workers (Holtz, 1929; Reerink and van Wijk, 1929) that the exclusion of short waves during irradiation is desirable. This is not, however, essential, except for obtaining the maximum yield of calciferol. It is clear that, at least with "long wave" radiation, the yield of crude calciferol is sufficient to account for most, if not all, of the observed activity of the crude irradiation products. These had an antirachitic activity of 22,000 International units per milligram in the example described above.

(*b*) *Oxygen.*—At first scrupulous care was taken to exclude oxygen throughout the process, but it is now found that, once irradiation is completed, the exclusion of oxygen is not necessary in the subsequent manipulation, unless distillation is intended.

(*c*) *Removal of Ergosterol.*—On some occasions the removal of the last traces of ergosterol by digitonin was only carried out after esterification and hydrolysis. As this necessitated a second esterification to get a pure product, this procedure is not recommended.

(*d*) *Esterification.*—There is reason to think that impurities in the dinitrobenzoyl chloride may cause some diminution in yield. Our own samples were prepared by refluxing the acid with 7 parts of thionyl chloride for 3 to 4 hours. The excess of thionyl chloride was removed by distillation, and the residue crystallised from a little benzene.

The period of heating during esterification can be reduced to 10 or even to 5 minutes without seriously affecting the yield, and a cleaner product is obtained.

Properties of Calciferol.

Calciferol thus purified forms clusters of colourless needles of melting-point 114·5° to 117°, $[\alpha]^{20}_{5461}$ + 122°, and $[\alpha]^{20}_{D}$ + 102·5° in alcohol. In order to facilitate comparison of the products described in this paper their physical constants are summarised in Table II.

Calciferol shows intense ultra-violet absorption, giving a smooth curve with a maximum at 265 mμ of 48·5 (= 2·25 in the units used by Windaus) as shown in fig. 1.* It is very soluble in most organic solvents, but may be recrystallised from 5 parts of methyl alcohol or 3 parts of acetone. It is not

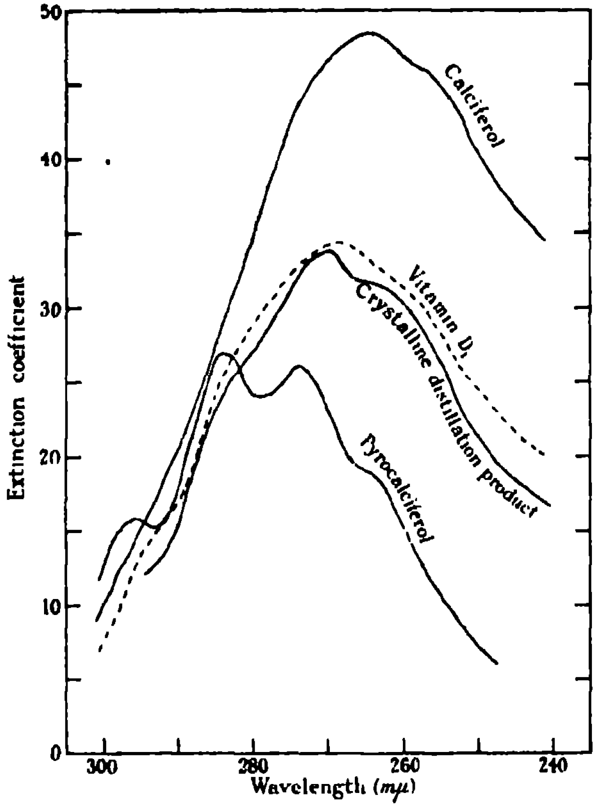

FIG. 1.—Absorption Spectra of Calciferol, Vitamin D_1, Crystalline Distillation Product, and Pyrocalciferol.

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* The absorption spectra were measured by the spectrophotometric technique described in our previous papers. The extinction coefficients are calculated by the formula

$$\varepsilon = (\log_{10}I_0 - \log_{10}I_1 - K)/Cd,$$

where I_0 and I_1 are the intensities of light before and after traversing *d* cm. of a solution of concentration C grams per litre, and K is the value of $\log_{10}I_0/I_1$ when the absorption cell contains solvent alone. The coefficients thus obtained can be converted to the units used by Windaus by multiplying by $c/0\cdot4343$ where *c* is the percentage concentration used by Windaus.

precipitated by digitonin. Elementary micro-analysis gave the following results :—

	Calciferol.		Calciferyl dinitrobenzoate.	
	Observed.	Calculated for $C_{27}H_{42}O$.	Observed.	Calculated for $C_{34}H_{44}O_6N_2$.
C	84·5	84·7	71·1	70·8
H	11·1	11·1	7·0	7·7

The apparent molecular weight of the dinitrobenzoate, as found by freezing-point depression in benzene, was 571 (calculated 576). Titration with perbenzoic acid indicated the presence of three double linkages (observed value 3·3). From these data it appears that calciferol is an isomeride of ergosterol.

Colour Reactions.—Table I shows the colour reactions of calciferol and also of pyrocalciferol and sterol X. The blue colouration given by calciferol with the Tortelli-Jaffé reagent is unusual.

Table I.—Colour Reactions.

Reagent.	Calciferol.	Pyrocalciferol.	Sterol X.
Liebermann-Burchard	Red → purple → emerald (stable).	Yellow→ deep blue which fades on standing.	Red → purple → emerald (stable).
Antimony trichloride	Deep yellow.	Colourless ; pale blue-green on standing.	Pink→ pale yellow.
Trichloroacetic acid	Deep yellow→ dull, dirty red-brown.	Colourless ; pale blue-green on standing.	Pink → purplish blue.
Mercuric acetate in nitric acid	In concentrated solution $CHCl_3$ layer orange-red → yellow ; acid yellow.	$CHCl_3$ layer momentary yellow ; acid yellow.	$CHCl_3$ layer carmine → buff.
Tortelli-Jaffé	Blue	Green	Green.

Effects of Ultra-violet Radiation.—Both " long waves " and " short waves " cause a rapid transformation of calciferol into products showing little absorption at wave-lengths greater than 260 mμ.

The term " long waves " is used in this paper for the radiation from a mercury arc filtered through xylene, which cuts off wave-lengths shorter than 275 mμ.

" Short waves " refers to radiation filtered through chlorine and bromine, and consisting mainly, but not entirely, of wave-lengths between 210 mμ and 280 mμ (Askew, *et al.*, 1930, *b*).

Antirachitic Activity.—This was determined by the radiographic method described in a recent monograph (Bourdillon *et al.*, 1931). Forty rats were used for each test. Of these, 20 were given doses of a standard solution of irradiated ergosterol in olive oil, and 20 litter-mates were given doses of the test substance also dissolved in oil. The results are expressed in terms of the International Unit defined at the League of Nations Conference in June, 1931 (League of Nations, 1931). This unit is the " vitamin D activity of 1 mg. of the International Standard Solution of irradiated ergosterol," and has the same value as the Medical Research Council Unit used in our previous paper.

Six different specimens of calciferol gave the following results, 40,400, 36,400, 37,000, 47,600, 38,300, 43,200 International Units per milligram. The mean of these six tests is 40,483, which is close to twice the recorded value of 20,000 units for the calciferol previously described as obtained by distillation. An estimate of the accuracy of these tests was made by examining the standard deviations for 1000 of the rats used during the period covered by this work, and showed that, for tests based in each case on 20 pairs of rats, the error corresponding to twice the standard deviation, *i.e.*, the error expected to be reached or exceeded only once in 22 tests, was plus 25 per cent. or minus 21 per cent. The probable error (or error expected once in two tests) is 8 per cent. These values are in close agreement with those calculated for earlier tests in the monograph referred to above.

Toxicity.—This was determined by the method of Moll (Holtz *et al.*, 1931), using mice. As defined in this method the toxic dose is the minimum daily dose, that kills or causes a loss in weight of 2·5 g. in the mice used during a period of 12 days. We gave the required dose always in a volume of 20 cu. m. m. of olive oil, which was administered directly into the animals' mouths. Four mice were used for each dose. The results are given in detail, since it was only possible to carry out a small number of tests.

Calciferol (of antirachitic activity 40,000 *units per milligram).*

0·2 mg.	....	1 dead, 2 severe loss in weight, 1 normal.
0·1 mg.	....	2 severe loss in weight, 1 slight loss, 1 normal.
0·05 mg.	....	No loss in weight.

Crude calciferol obtained by distillation (of antirachitic activity 20,000 *units per milligram).*

0·4 mg.	....	1 dead, 3 severe loss in weight.
0·2 mg.	....	1 severe loss, 2 slight, 1 normal.
0·1 mg.	....	No loss in weight.

Vitamin D_1 *(Professor Windaus's sample) (of antirachitic activity about* 20,000 *units per milligram).*

0·4 mg.	....	2 dead, 2 severe loss in weight.
0·2 mg.	....	2 severe loss in weight, 2 slight.
0·1 mg.	....	No loss in weight.

The minimal toxic dose is seen to be of the order of 0·1 mg. for calciferol; 0·2 mg. for crude calciferol; and 0·2 mg. for vitamin D_1. This is equivalent to about 4000 International antirachitic units in each case. Thus the ratio " toxic dose " (in mice) to " curative dose " (in rats) is about 4000 to 1.

Although the number of tests is too small to be conclusive, these results support the view that the toxicity is an inherent property of the pure vitamin, and is not due to a contaminant (*cf.* Harris and Innes, 1931; Windaus *et al.* 1931, *b*).

Calciferyl p-*Nitrobenzoate.*—This compound was prepared by warming a mixture of calciferol and *p*-nitrobenzoyl chloride in pyridine. It formed pale yellow crystals from alcohol, melting-point 90° to 93°, $[\alpha]^{20}_{5461}$ + 65° in benzene. Hydrolysis yielded calciferol of normal physical properties and antirachitic activity.

Pyrocalciferol.

As previously recorded (Askew *et al.*, 1931), a compound of very high dextrorotation is formed when certain irradiation products of ergosterol are heated to 180°. The name pyrocalciferol was proposed for this compound owing to its origin and association with calciferol. It was prepared as follows from the crude calciferol obtained by distillation. This material was esterified by heating for a few minutes to 100° with an equal weight of 3 : 5-dinitrobenzoyl chloride dissolved in pyridine. The product was then freed from pyridine by washing repeatedly with water. The tarry residue was washed with alcohol, and dissolved in a little benzene. Addition of 5 parts of alcohol to the solution precipitated pale-yellow microscopic needles, which were removed immediately and washed with a little alcohol. These were then recrystallised repeatedly from acetone, from which pyrocalciferyl 3 : 5-dinitrobenzoate separated as orange-coloured prisms, melting-point 166° to 169°, $[\alpha]^{20}_{5461}$ + 250° in benzene. This ester was hydrolysed by the addition of a slight excess of 2N aqueous sodium hydroxide solution to a suspension of the solid ester in boiling alcohol. The product crystallised from 10 parts of methyl alcohol in thin colourless needles.

Properties.—Pyrocalciferol has been obtained with melting-point 93° to 95°, $[\alpha]^{20}_{5461}$ + 624°, and $[\alpha]^{20}_{D}$ + 502° in alcohol, although the purification has not been carried far enough for these data to be regarded as final. The dextrorotation is very high, and probably accounts for the large increase in total dextrorotation that is generally observed in the distillation of the irradiation products of ergosterol. The absorption spectrum is shown in fig. 1. It is closely similar to that of ergosterol, but the maxima are at longer wavelengths. There is reason to think that it is not present in normal irradiation products of ergosterol, but is formed from one of them during distillation at 150°.

Antirachitic Activity.—Pyrocalciferol appears to have no antirachitic activity. Tests show that daily doses as large as 0·01 mg. are without perceptible effect, and hence, if active at all, its activity is less than 1/800th of that of calciferol.

Colour Reactions.—These are given in Table I. The blue colouration given with the Liebermann-Burchard reagent is noteworthy.

Effects of Ultra-violet Radiation.—Both " long waves " and " short waves " convert pyrocalciferol into products of little absorption, but it is transformed much less rapidly than calciferol.

Analysis.—Elementary micro-analysis gave the following results :—

	Pyrocalciferol.		Pyrocalciferyl : dinitrobenzoate.	
	Observed.	Calculated for $C_{27}H_{42}O$.	Observed.	Calculated for $C_{34}H_{44}O_6N_2$.
C	84·7	84·7	71·1	70·8
H	11·2	11·1	7·7	7·7

The apparent molecular weight, as found by freezing-point depression in benzene, was 544 for the dinitrobenzoate (calculated 576). Titration with perbenzoic acid indicated the presence of either two or three double linkages (observed values 2·5 and 2·6). The formation of an ester, and these analytical data, suggest that pyrocalciferol is closely related to ergosterol, and is probably isomeric with it.

Pyrocalciferyl 3 : *5-dinitrobenzoate.*—This ester forms orange-coloured prisms from acetone (with the properties given above). It dissolves readily in benzene and is precipitated by the addition of alcohol to the solution, as light yellow needles containing one molecule of benzene of crystallisation. One such specimen showed melting-point 154° to 161° (sealed capillary tube ; after softening at 135°) and $[\alpha]^{20}_{5461} + 219°$, which is equivalent to 249° for solvent-free material. When heated for 3 hours to 100° at 10 mm. pressure, it lost 11·4 per cent. of its weight, in good agreement with the calculated figure of 11·9 per cent., which corresponds to one molecule of benzene. On crystallisation from acetone the normal orange-coloured form free of solvent was again obtained.

Preparation of Sterol X.

As mentioned above, vitamin D_1 can be split up into calciferol and sterol X by esterification with 3 : 5-dinitrobenzoyl chloride. This process was applied to a specimen of vitamin D_1 made by ourselves by Windaus's method (Windaus *et al.*, 1931, *a*), using citraconic anhydride, and also to 0·25 g. of a specimen of vitamin D_1 which was kindly placed at our disposal by Professor Windaus. Calciferyl dinitrobenzoate separated from both preparations as the least soluble fraction, and there was obtained from the mother-liquors a second dinitrobenzoate separating in clusters of pale yellow needle-shaped crystals. After recrystallisation these had melting-point 139° to 141° and $[\alpha]^{20}_{5461} + 24°$ in benzene. The combined yields of this second dinitrobenzoate from the two preparations gave on hydrolysis sterol X, which separated as needles from methyl alcohol.

Properties of Sterol X.

The imperfectly purified sterol X forms crystalline needles of melting-point 116·5° to 118·5°, $[\alpha]^{20}_{5461} + 220°$, and $[\alpha]^{20}_{D} + 176°$ in alcohol. It is readily soluble in organic solvents, and is not precipitated by digitonin. It is believed to be without antirachitic activity, as the sample tested by us showed none in the largest doses used. If it does possess activity, this is certainly less than 1/30th that of calciferol. The colour reactions are given in Table I. It will be noticed that pink and carmine colourations are given with trichloroacetic acid, and with mercuric acetate in nitric acid, indicating the presence of a $\Delta^{1,2}$ (or $\Delta^{1,13}$) double linkage (Rosenheim, 1929), which is present in ergosterol, but absent in calciferol and pyrocalciferol.

Absorption Spectrum and Effects of Irradiation.—The absorption spectrum of this substance is shown in fig. 2, together with that of the same product after exposure to "short wave" ultra-violet irradiation (for 10 minutes in 0·001 per cent. solution in a layer 1 cm. thick, 11 cm. from a mercury vapour lamp). The striking increase in absorption at 280 mμ makes it probable that

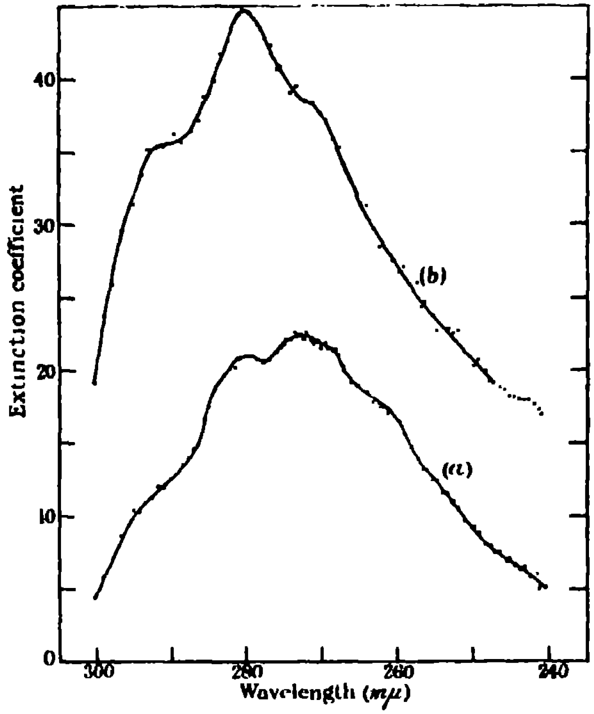

Fig. 2.—(*a*) Absorption Spectrum of Sterol X. (*b*) Same after 5 minutes' exposure to "short waves."

sterol X is identical with the compound referred to in a previous paper (Askew *et al.*, 1930, *b*) on the effects of further irradiation on the irradiation products of ergosterol. As there pointed out, it is probable that sterol X is formed simultaneously with vitamin D (calciferol) as a direct irradiation product of ergosterol. On exposure to "long waves" sterol X is slowly converted into products showing little or no absorption above 260 mμ.

Relation of Calciferol to other Crystalline Antirachitic Substances.

(*a*) *Calciferol and Vitamin D$_2$.*—The few particulars of the physical properties of vitamin D$_2$ already published (Windaus *et al.*, 1931, *a* and *c*) are in fairly close agreement with those of calciferol. Our physical measurements of the specimen of vitamin D_2 which Professor Windaus courteously sent to us, again show fairly good agreement with those of calciferol, although the values are slightly lower, probably because the sample, as indicated, was "not quite pure." A mixture of the two substances shows no depression of melting point. The antirachitic activity was found to be 44,600 units, and thus agreed with that of calciferol within the limits of error of the test. In addition, Windaus's specimen of the dinitrobenzoate of vitamin D_2 possessed physical constants similar to those of calciferyl dinitrobenzoate, and a mixture of the two esters showed no depression of melting point. The relevant physical measurements are given in Table II. There is little doubt that the two substances are identical, as Windaus has already concluded in an addendum to his latest paper (Windaus *et al.*, 1931, *c*).

(*b*) *Calciferol and Vitamin* D_1.—We are in full agreement with Windaus's conclusion that vitamin D_1 is an addition compound of vitamin D_2 (calciferol) and an isomeric alcohol. As mentioned above, we also have obtained this alcohol, and have described it under the provisional name of sterol X. The separation of vitamin D_1 into these constituents has thus been carried out in both laboratories, and is the chief evidence of its composition. Further confirmation is given by absorption measurements taken in dilute solution, where molecular association appears to be negligible. In fig. 3, A, the continuous curve shows the observed absorption of a specimen of vitamin D_1 prepared by Windaus's method in this laboratory. The broken curve shows the values for an equimolecular mixture of calciferol and sterol X, as calculated from the curves for the separate compounds. The two curves agree closely. The optical rotation of vitamin D_1 in dilute solution is very close to the mean of the rotations of the separate compounds, thus confirming their presence in equimolecular proportions. Similar evidence is given by the measurements of antirachitic activity, which show that vitamin D_1 has approximately half the activity of calciferol.

(*c*) *Calciferol and the Crystalline Distillation Products described formerly.*—After the publication of our account of the crystalline product of distillation (Angus *et al.*, 1931), we obtained, by varying the conditions of distillation, a number of specimens which, on repeated recrystallisation, attained constant but differing optical rotations. Although these rotations varied from $[\alpha]^{20}_{5461}$ + 220° to + 325°, the antirachitic activities of all specimens were very nearly equal. It is now believed that all these specimens consisted of solid solutions of the association compound (= vitamin D_1) of calciferol and sterol

Table II.—Properties of Calciferol, Allied Compounds, and their Esters.

Substance.	Melting-point.	$[\alpha]^{20}_{5461}$.		$[\alpha]^{20}_{D}$.		Maximum absorption.		Antirachitic activity (International Units per mg.).
		Ethyl alcohol.	Acetone.	Ethyl alcohol.	Acetone.	ε.	λ.	
Sterols—	°C.	°	°	°	°		mμ.	
Purified calciferol	114·5–117	+122·5	+ 98·5	+102·5	+ 81	48	265	40,483 (mean)
Vitamin D_2 (*a*)	114 –115	—	—	—	+ 85	47	265	(*h*)
„ (*b*)	110·5–113·5	+120	—	—	—	43	263	44,600
Vitamin D_1 (*c*)	124 –125	+174	—	+141	—	34	268	—
„ (*d*)	124 –127	+176	—	—	+136·3	34	268	23,700
Pyrocalciferol	93 –95	+624	—	+502	—	16 27 26	296 284 274	—
Sterol X	116·5–118·5	+220	—	+176	—	21 22·5	280 273	—
Crystalline distillation products (*e*)	123 –125	+260	—	—	—	34	270	20,000 (mean)
Crystalline distillation products (*f*)	119 –123 to 121 –125	+220 to 325	—	—	—	33	270	20,000 (mean)
Calciferol + pyrocalciferol 1 : 1	120 –122·5	+374 (observed) 373 (calculated)	—	—	—	35 (observed) 36 (calculated)	272 273	— —
3 : 5-*dinitrobenzoates*—								
Of calciferol	147 –149	In benzene + 69·5	+105	—	—	—	—	—
Of vitamin D_2 (*g*)	145 –147	+ 70·5	+105·5	—	—	—	—	—
Of pyrocalciferol	167·5–169·5	+250	—	—	—	—	—	—
Of sterol X	139·5–141·5	+ 24	—	—	—	—	—	—

(*a*) Recorded by Windaus *et al* (1931, *a*).
(*b*) Specimen from Professor Windaus, labelled "not quite pure."
(*c*) Specimen prepared by us.
(*d*) Specimen from Professor Windaus.
(*e*) Recorded by Askew *et al.* (1931, *a*).
(*f*) Later specimens.
(*g*) Specimen from Professor Windaus.
(*h*) "Etwas wirksamer als das Vitamin D." (Windaus *et al*, 1931, *c*).

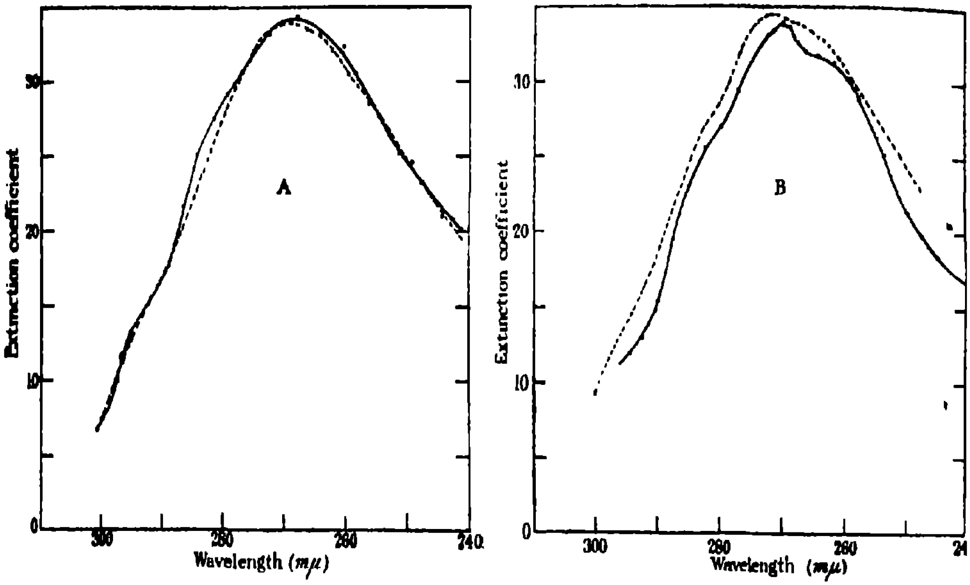

Fig. 3.—Observed and calculated absorption spectra of (A) vitamin D_1 and (B) crystalline distillation product. (A) Broken curve, calculated for 50 per cent. calciferol, 50 per cent. sterol X. Continuous curve, observed for Windaus's vitamin D_1. (B) Broken curve, calculated for 50 per cent. calciferol, 21 per cent. pyrocalciferol, 29 per cent. sterol X. Continuous curve, observed for crystalline distillation product $[\alpha]^{20}_{5461}$ + 260°.

X, and another association compound of equimolecular proportions of calciferol and pyrocalciferol. The evidence for this is as follows :—

(1) When such a preparation is esterified with 3 : 5-dinitrobenzoyl chloride three separate esters can be obtained, and identified by their physical properties as those of calciferol, pyrocalciferol, and sterol X.

(2) As shown in fig. 3, B, the absorption curve of the crystalline distillation product corresponds fairly closely to the composite curve of a mixture of these three substances in the proportion calculated from its optical rotation. The difference between the two curves is within the error of measurement, but may be due to a contaminant.

(3) Mixtures of calciferol and pyrocalciferol in various proportions were made up in alcohol, and crystallised at 4° C. after the addition of water. The composition of the crystals was calculated from their specific rotation when redissolved in alcohol (the rotations of mixtures of these two substances having been shown to be strictly additive in 0·5 per cent. solution). The melting-points of these crystals are plotted against

composition in fig. 4. The presence of a central maximum, and at least one eutectic point, affords clear evidence for a 1 : 1 molecular compound of calciferol and pyrocalciferol. The existence of a series of solid solutions formed by vitamin D_1 and the calciferol-pyrocalciferol compound is indicated by the fact that a mixture of the two shows no depression of melting-point. Mixtures of either with the crystalline distillation product show no depression. Further, a synthetic mixture of vitamin D_1 and calciferol-pyrocalciferol having $[\alpha]^{20}_{5461}$ + 259° and melting-point 122·5° to 125°, crystallised from aqueous alcohol, showed no depression with any of the three. Depressions of melting-point, which indicate the existence of eutectics, are shown by mixtures of vitamin D_1 and sterol X, and of calciferol and crystalline distillation products. Hence there is reason to believe that the two molecular compounds can form a series of solid solutions, all containing 50 per cent. of calciferol, and that the widely varying rotations, but constant biological activity, of the crystalline distillation products can be accounted for by assuming that they are all members of this series.

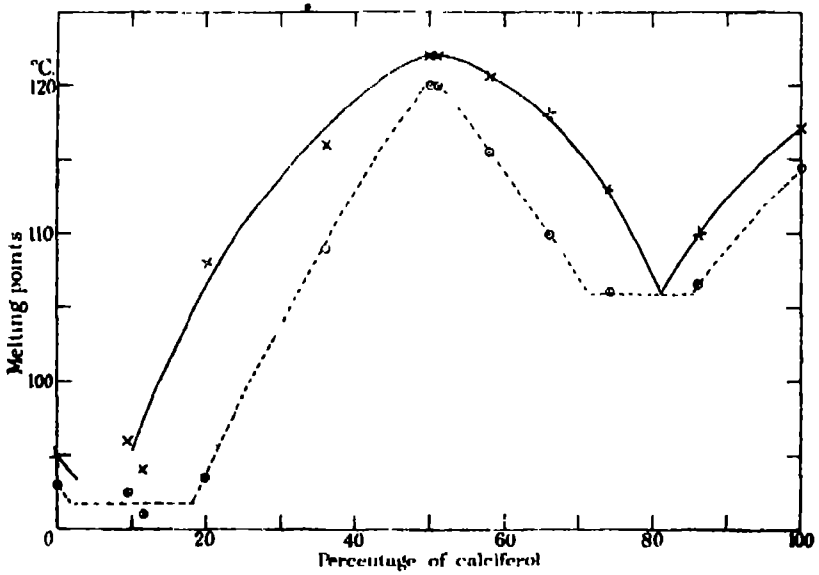

FIG. 4.—Melting-points of mixtures of calciferol and pyrocalciferol. The upper curve represents complete fusion, the lower curve incipient fusion.

(*d*) *Calciferol and "Substance L."*—We have not been able to study the crystalline "substance L" described by Reerink and van Wijk (1931, *a* and *b*), but we venture to suggest that it is a mixture containing calciferol and one or more inactive irradiation products.

Evidence as to the Purity of Calciferol.

There is considerable evidence for the belief that the calciferol now obtained is a nearly pure single compound. Thus, different samples of calciferol show but little variation in physical properties, whether prepared from the irradiation products of ergosterol by direct esterification, or by the removal of contaminants by citraconic anhydride, or by distillation and subsequent esterification. Reasonable constancy is also shown by the antirachitic activity, if allowance is made for the errors to which these measurements are subject. Further, calciferol gives crystalline esters such as the dinitrobenzoate and *p*-nitrobenzoate, which, on recrystallisation and hydrolysis, give back the original sterol with unchanged physical and biological properties. The fact that it is readily separated as the dinitrobenzoate, from addition compounds with pyrocalciferol or sterol X, makes it improbable that calciferol is itself an addition compound.

It is impossible to secure complete proof that the activity of calciferol is not due to small quantities of an intensely active contaminant, but this is rendered most improbable by the ease with which closely allied sterols, such as pyrocalciferol, can be separated without perceptible antirachitic activity. Three known possible contaminants which can be excluded are sterol X, and its "short wave" irradiation product, and pyrocalciferol. Their absence is shown by the rapid and complete disappearance of absorption in the region 270–300 mμ when calciferol is irradiated with "short waves." The absence of pyrocalciferol is also shown by the identical rotation of calciferol obtained from heated and unheated products.

In any ordinary class of compounds the evidence thus obtained would be satisfactory proof of purity. The sterols, however, are a group in which the frequent occurrence of association compounds and solid solutions renders it very difficult to be certain of the purity of a new compound. Hence, until an exhaustive purification with large quantities of material can be undertaken, we can only say that calciferol is probably a simple compound in a state of approximate purity.

Summary.

Further purification of the antirachitic principle has been achieved, by esterification of the crystalline distillation products formerly described as calciferol. The purified calciferol now obtained has an antirachitic activity twice as great as any previously recorded, and appears to be identical with the vitamin D_2 of Linsert and Windaus.

The purified product still produces toxic effects when given in excessive doses.

A simple process is described for preparing the pure product from the irradiation products of ergosterol without distillation.

An account is given of two allied inactive compounds termed pyrocalciferol and sterol X (= "Lumisterin").

Evidence is given as to the relations between the crystalline antirachitic products hitherto described, showing that the activity of each of them is due to one common constituent. Thus, the vitamin D_1 of Windaus is a compound of calciferol (= vitamin D_2) and sterol X, and the crystalline distillation products formerly described contain pyrocalciferol and sterol X, as well as calciferol.

The probability is discussed that calciferol (= vitamin D_2) represents "vitamin D" in a state of approximate purity.

We are indebted to our colleague, Mr. H. P. Marks, for the micro-analyses recorded in this paper, and Dr. E. Schuster, for the design and construction of much of the apparatus used for absorption measurements.

We wish to thank Dr. H. H. Dale and Dr. O. Rosenheim for help in the revision of this paper. We also wish to thank Dr. Dale for personal encouragement throughout this work.

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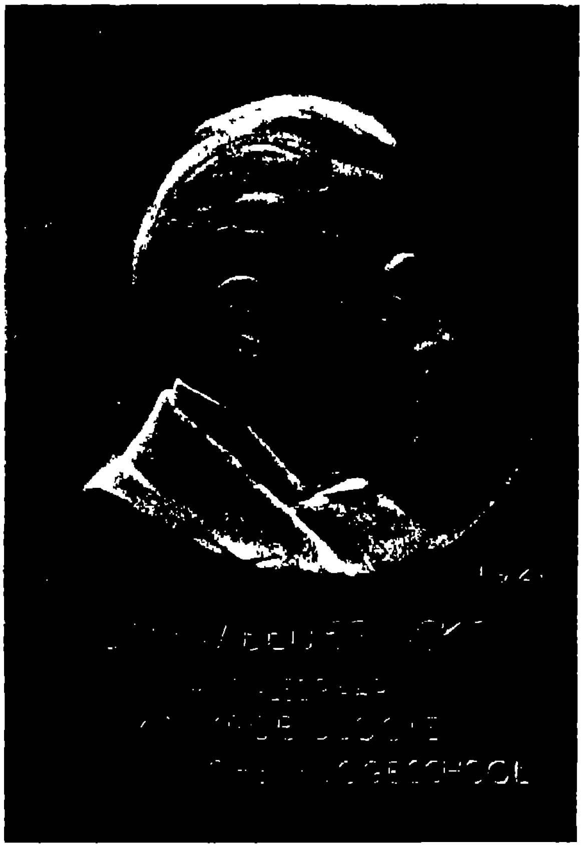


M W Beyerinck

MARTINUS WILLEM BEIJERINCK—1851-1931.

AT all periods Holland has played a conspicuous part in the development of science, and in more than one branch of learning Dutchmen have led the path of advance. The Royal Society has not been wanting in its appreciation of their efforts and honoured names like Huygens, Leeuwenhoek, Ruysch, Boerhaave, Donders, de Vries, van't Hoff, Zeeman and Lorentz will be found on its roll. To this galaxy must be added the name of Martinus Willem Beijerinck, botanist, microbiologist and chemist, who in the course of researches steadfastly prolonged for half a century gave abundant proof of high scientific genius and practical achievement.

Martinus Willem Beijerinck was born in Amsterdam on March 16, 1851, the son of a tobacconist. On the paternal side his grandfather was an engineer of the Waterstaat, while his maternal grandfather was a Protestant pastor and learned theologian. The tobacconist father had the artistic rather than the business temperament, gave up his shop and settled in Haarlem, where he managed to secure a post on the railway. Martinus went to the Hoogere Burgerschool in Haarlem, and early showed such an interest in the wild plants of the surrounding country that at the age of sixteen he received a silver medal from the Dutch Society of Agriculture for an essay on the flora of Kennemerland, and so great had become his love for botany that he thought of adopting it as a career. Financial straits, however, made him look to a more assured future, and in 1869 he entered the Polytechnic Institute at Delft, where, in 1872, he took the diploma of "Chemical Engineer." For a short time he returned to his botanical studies in Leyden, but in 1873 he took up a post as lecturer on botany, physiology and physics at the Agricultural School at Warffum, not far from Groningen.

In 1875 he was lecturer on botany, zoology and geology at the Hoogere Burgerschool at Utrecht, and from there he went in 1876 as lecturer in botany at the Agricultural School at Wageningen, which soon afterwards was transformed into the Rijks hoogere Land-, Tuin- en Boschbouwschool, and there he remained for ten years, during which his great scientific gifts displayed themselves to the full. In 1877 he took the degree of Doctor in Wis- en natuurkunde in Leyden with a dissertation, "Bijdrage tot de morphologie der plantengallen," and he continued to interest himself in this subject for many years.

In 1887 he was offered and accepted the post of microbiologist to the "Nederlandsche Gist-en Spiritusfabriek" in Delft, and, in this small and delightful town of historic, artistic and scientific memories, Beijerinck spent the next thirty-four years of a peaceful and outwardly uneventful life, diligently employing his great talents for the advancement of biological science.

In 1893 he became professor of general bacteriology and, in 1895, of general microbiology at the Technische Hoogeschool in Delft, and there he founded a laboratory which became famous all over the world. Having attained the age of seventy in 1921, he retired from his professorship in Delft, settled in Gorssel, a small town in Gelderland near Deventer, and there he died, deeply regretted, on January 1, 1931, aged seventy-nine. He left instructions that no funeral orations were to be delivered over his remains, which were cremated on January 6.

Between 1877 and 1927 Beijerinck published about 140 scientific researches, each of which contained evidence of original work, and many have become classical. His papers are scattered and appeared in Dutch, French, German and English periodicals. It is fortunate that most of them have been gathered together in a series of five large quarto, closely-printed volumes, published to commemorate his seventieth birthday. This notable publication appeared from Delft in 1921, and is entitled, "Verzamelde Geschriften van M. W. Beijerinck ter gelegenheid van zijn 70sten Verjaardag."

In reading through this large work, one is struck not only with Beijerinck's originality but also with the extraordinary range of his observations, for he was equally at home as a botanist, microbiologist, and chemist. The subjects he chose to work at were of great importance, theoretically and practically, and, although they were often of great complexity, he was peculiarly successful in getting at the truth.

In 1877 he published an elaborate work on plant galls, and for this he was early elected a member of the Koninklijke Academie van Wetenschappen. In 1882 he discovered that gummosis in the cambium of Prunaceæ is an infective process caused by the widespread fungus *Coryneum beijerinckii* Oudemans, and with this fungus he succeeded later in obtaining large quantities of gum from the cherry, apricot, plum and especially *Prunus amygdalo-persica.* He also made a number of experiments in crossing different varieties of wheat.

In 1888 he published in German his classical paper, "Die Bacterien der Papilionaceen-Knöllchen," and gave an elaborate and accurate account of the origin, structure, and fate of the nodules. He showed that the process was the result of an infection caused by *Bacillus radicicola*, which he succeeded in cultivating from the nodules.

From about 1889 he began to turn his attention more and more to economic and soil bacteriology, and by his notable contributions made himself the recognised master in this complicated but important branch of bacteriology. By studying the fundamental problems of bacterial nutrition, he was successful in cultivating many bacteria where others had failed, and he was early in the field in investigating several subjects which may be described as all the rage to-day.

Special reference must be made to his extended and beautiful researches on luminous bacteria and the mechanism of luminosity and the respiratory figures of motile bacteria. He brought to light the extraordinary oligonitrophilous and oligocarbophilous bacteria and the important aerobic nitrogen-fixing *Azotobacter chroococcum*. Beijerinck also wrote a great deal on the chemistry of bacterial fermentations and was a successful and early worker on the hereditary variations among bacteria. His researches on enzymes such as lactase, glucase and tyrosinase were elaborate, and he spent much time in the elucidation of the natural indigo-forming process.

In 1898 he described the filter-passing character of the infective agent in the tobacco blight, now called mosaic disease. In this discovery he had been forestalled by Iwanowski, who had reported the filter-passing character of the virus in 1892. Iwanowski's claims were handsomely admitted by Beijerinck in his full account of the subject in his paper, " De l'existence d'un principe contagieux vivant fluide, agent de la nielle des feuilles de tabac," published in 1900.

In the midst of all these strenuous labours, Beijerinck would return to such subjects as the sexual generation of *Cynips Kollari*, the assimilative powers of the chromatophores of diatoms, the influence of very low temperatures on bacteria, the enzyme theory of heredity, crystallised starch and the chemistry of indigo. His interests seemed inexhaustable, and he touched no subject that he did not enrich with his genius and industry. He worked quietly and unobtrusively as the years sped on, but even in his retirement at Gorssel his scientific interest was unabated, and in his last days he was again a botanist working at the mathematics of phyllotaxis.

Judged by the severest standards, Beijerinck's scientific labours and achievements occupy a high place and entitle him to rank alongside the greatest biologists of the twentieth century. In his own country he was held in the highest repute, and was awarded the Leeuwenhoek medal in 1905 by the Koninklijke Akademie van Wetenschappen. In 1922 he received the Emil Christian Hansen medal from Denmark. Beijerinck was an honorary member of many biological societies outside Holland, and he wrote to me that the crowning pleasure of his life was his election as Foreign Member of the Royal Society in 1926.

Besides his great scientific achievements, Beijerinck was a simple and noble character and habitually displayed the most generous instincts, not only towards his own countrymen, but also to foreigners who corresponded with him. To perpetuate his memory a fine bronze plaque with his likeness was placed, in 1921, in the Technische Hoogeschool in Delft, where he taught and laboured so successfully for more than a quarter of a century. This plaque is herewith reproduced.

W. B.

JAMES LORRAIN SMITH—1862-1931.

PROFESSOR LORRAIN SMITH, who died on April 18 of this year, was elected a Fellow in 1909, and served on the Council in 1913-14. He was born in 1862 at Half Morton, Dumfriesshire, where his father was for nearly forty years the greatly loved and respected Free Church Minister of the small parish. There were several brothers and sisters, who were brought up in an atmosphere of culture and kindly human relationships, and most of whom lived to distinguish themselves.

Lorrain Smith was educated at George Watson's College, Edinburgh, and Edinburgh University. He graduated as M.A. with First-Class honours in Philosophy in 1884, and as M.B., C.M. in 1889. During his medical course he gained the Vans Dunlop Scholarship in Logic and Metaphysics, and the Ferguson Philosophical Scholarship, open to students of all the Scottish Universities. It was in connection with the University Philosophical Society that one of us first became friends with him; and throughout his life his interest in the philosophical aspects of biology was unabated. Just before the sudden bad turn in the illness which caused his death he was looking forward to completing a book on "Growth," in which his wider conclusions were to be embodied.

On completing his medical course he went to Oxford, to study and work in Burdon Sanderson's laboratory. Here he took the recently organised advanced courses and at the same time began research work in conjunction with one of us on the physiological action of air vitiated by respiration. The results were published in the Journal of Pathology (1892 and 1893) and showed (1) that there is no basis for the idea then current that a harmful volatile organic product is present in expired air, (2) that the hyperpnoea and other symptoms produced before the oxygen percentage becomes very low are due entirely to the carbon dioxide in the air, and (3) that certain characteristic symptoms are produced by want of oxygen, in the absence of excess of carbon dioxide.

In the summer of 1890 he was Resident Physician at the Edinburgh Royal Infirmary, and in the succeeding summer worked with von Recklinghausen at the Strassburg Pathological Institute. Thereafter he acted for a time as an Oxford University Extension Lecturer, and later as an Assistant Demonstrator in Physiology, but continuing all the time his research work, and meanwhile starting independent work on the general metabolism after thyroidectomy in animals. This work (published in 1894) showed that after thyroidectomy the general metabolism does not vary in a normal manner in response to changes in external temperature, so that the normal process of regulation of body-temperature is disturbed. In conjunction with Dr. Pembrey, he also published an account of the histological changes after thyroidectomy.

In 1893 he was appointed John Lucas Walker Student at Cambridge, and went to work there under Roy, helping Kanthack with the courses in bacteriology, and finally becoming Demonstrator in Pathology, and also lecturing to advanced students on the physiology of respiration and animal heat. During the Christmas vacation of 1893, however, he and Haldane went to work in Christian Bohr's laboratory at Copenhagen, in order to get a thorough grasp of the existing methods of blood-gas investigation. A piece of research which they carried out there was, unfortunately, vitiated through defects in the determination of iron. In 1894 he was appointed Lecturer in Pathology at Queen's College, Belfast, and took up work there next summer after his marriage to Isabella Edmond, the devoted wife who helped him so much throughout his career and largely contributed to its success.

During the following Christmas vacation he and Haldane carried out a careful series of experiments on the arterial oxygen-pressure during rest in man, using a new method dependent on the relative affinities of oxygen and carbon monoxide for hæmoglobin. The results (1896) seemed to indicate very clearly that the mean oxygen-pressure of the blood leaving the pulmonary alveoli was higher than in the alveoli themselves, thus pointing to active secretion inwards. A further investigation on several kinds of animals in the following year gave confirmatory results. Later and still more careful experiments by Douglas and Haldane showed, however, that it is only when want of oxygen exists in body tissues that this active secretion occurs, and particularly after acclimatisation to want of oxygen. Want of oxygen was produced by the carbon monoxide used in the experiments on both men and animals; and in addition the two human subjects had become acclimatised through repeated exposures to carbon monoxide during the series of experiments. The same method showed that when these sources of disturbance are avoided there is, during rest, exact correspondence between oxygen pressure in the alveoli and in the blood leaving them. This fact does not diminish, but increases, the physiological interest and importance of the original observations.

He now extended the investigation to pathological states and found (1898) that in artificially produced fever the active secretion disappeared. Experiments by the same method with high pressures of oxygen led him then to the important discovery that exposure of the lungs to moderately high pressure of oxygen produced fatal pneumonia. It was already known from the experiments of Paul Bert that toxic effects on the central nervous system are produced when an animal breathes oxygen at more than about three atmospheres' pressure. The poisonous action on the lungs was a quite new discovery, and was shown to occur at an oxygen pressure of even less than one atmosphere.

A further paper in conjunction with Haldane (1900) describes a new method of measuring, with the help of carbon monoxide, the volume and total oxygen capacity of the blood in man during life. In this paper it was shown for the first time that the colouring power of the blood is exactly proportional to its capacity for taking up oxygen or carbon monoxide in loose combination. The new method was dependent on this fact. Only results for normal persons are given in the paper, but Lorrain Smith proceeded at once to apply it to pathological conditions. He found (1900) that in the so-called anæmia of chlorosis the volume of the blood is in actual fact greatly increased, but correspondingly diluted as regards hæmoglobin, so that the percentage of hæmoglobin is reduced in the characteristic manner, without reduction in the total hæmoglobin in the body. Some other anæmias are of this type, including that of ankylostomiasis, as was shortly afterwards found by Boycott and Haldane. On the other hand, pernicious anæmia, and that resulting from hæmorrhage, were not accompanied by increased blood-volume. He and McKisack also found that in a case of polycythæmia due to heart disease there was not only a great increase in the total oxygen capacity of the blood, but also a great increase in its volume. Other observers using the same method have found similar conditions in other conditions of polycythæmia, including that produced by life at high altitudes.

Meanwhile, he was called on to investigate the conditions on which an outbreak of typhoid in Belfast depended; and on this he did much valuable work, of which he published a summary in 1904. In 1901 his lectureship was made a professorship. He was also made a member of the Irish University Commission. From this time on he became more and more immersed in University affairs and in teaching, so that it was no longer possible for him to continue in the very exacting line of scientific work which he had been engaged in for the previous ten years. As the value of his wide knowledge and sympathies became more known, the calls on his time became greater and greater.

In 1904 he was appointed to the chair of Pathology at Manchester University, and he occupied this position with great profit to the University till 1912, when he was appointed to the chair of Pathology in Edinburgh University. It was in Manchester that he started that important series of investigations on fats and lipoids which were continued during the rest of his life. His first observation, namely, that when neutral fats are hydrolysed, *e.g.*, by carbonic acid, the free fatty acids combine with coloured bases and thus become stained, was followed by the examination of a large number of fats and dyes which led to the establishment of a general principle of staining fats with basic aniline dyes. The examination of one dye tested, Nile blue sulphate A, led to its being adopted as an important reagent for distinguishing acids and neutral fats in the tissues. Observing that when this dye was used, certain fatty globules in the tissues were stained red, he was able, with the aid of his colleague, Professor Thorpe, to show that this was due to a partial change of the oxazine base to an oxazone base, the latter staining neutral fat and also fatty acid owing to its marked solubility in them, while only acid fat was stained blue by the oxazine base. An extension of the enquiry to lipoids was the means of throwing fresh light on Weigert's method for staining nerve fibres and of demonstrating its general applicability as a staining method to fats. He showed that after a tissue was bichromated the staining, *i.e.*, the property of forming a coloured lake with hæmatoxylin, depends on the presence in the tissue of substances with unsaturated molecules such as alcohol or aldehydes. The time at which staining occurs depends upon a particular stage of oxidation and varies much in the case of different fats and lipoids. The myelin of nerve fibres rapidly becomes sufficiently oxidised, but if the bichromating is continued long enough it then ceases to stain ; neutral fat can be stained by Weigert's method, but the period of bichromating necessary is a long one. The ready staining of doubly refracting lipoids with a fluid crystalline phase also was investigated and was found to be often due to the presence of cholesterol combined with fatty acids. Further observations were carried on with regard to variations of the lipoids of the central nervous system at different periods of life and in conditions of degeneration, and important differences were shown to exist. Most of his papers from Manchester were written in association with W. Mair.

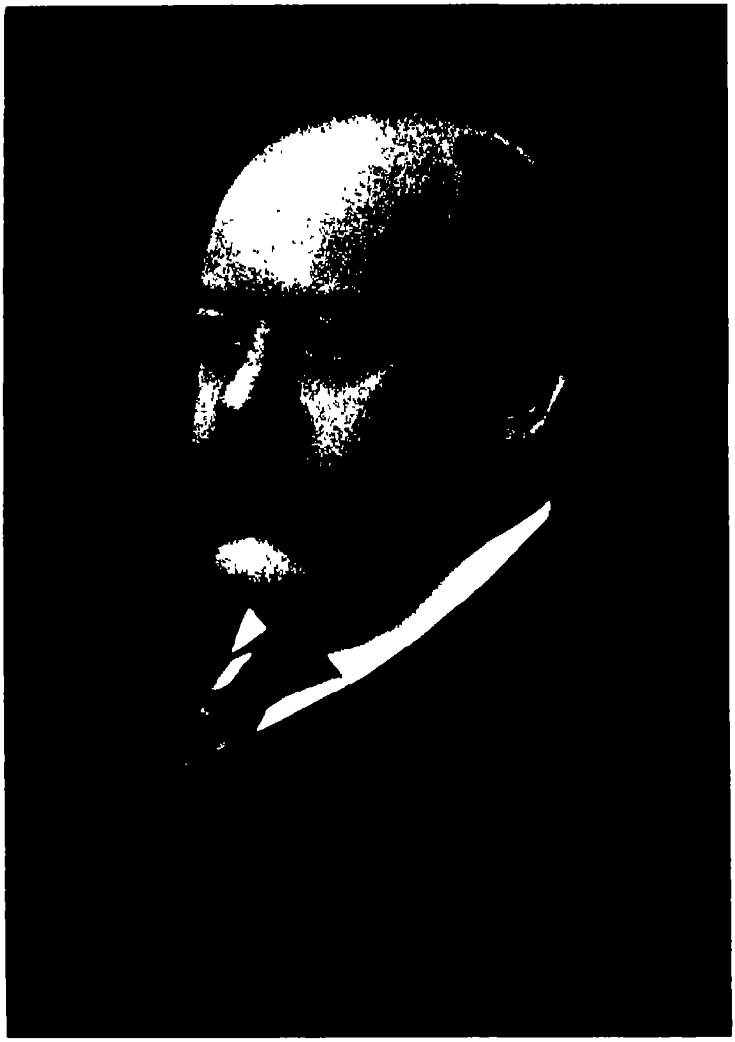
Lorrain Smith's work in this field was interrupted by his going to Edinburgh and then by the war, but was continued later in conjunction with Rettie. To this period belong papers on the mordanting of fats by aldehydes and their subsequent staining. The most suitable reagent for the purpose was found to be aldol, and it was shown that, after mordanting with this substance the granules and globules of fatty degeneration are readily stained, while store fat is not stained or stained only with difficulty. An important difference between the two kinds of fats was thus established and a ready method afforded for identifying degeneration fat. By use of the aldol method it was also shown that in cloudy swelling and in fatty degeneration the earliest change in cells is a destruction of mitochondria with a setting free of recognisable lipins. A contribution of striking character made by the same observers is that (published in the 'Proceedings,' 1927) on the demonstration of lymphatics by the result of autolytic change. It was here shown that in the process of autolysis the fats in the masked state in the lymph become hydrolysed and small globules of doubly refracting soaps are formed, which are so abundant in the liver as to form an injection of the lymphatics. The demonstration of this change was quite original. His last paper was on the absorption, deposition and transport of fat in the guinea-pig. In this the original observation is set forth that the fat absorbed from the intestine becomes deposited in the distal extremities of the lacteals as masses of neutral glyceride. The phenomenon is described as a process of storage, the fat being later dispersed according to the requirements of the body, and is related to the taking of food and its fat content. Of many animals examined the guinea-pig was the only one in which this phenomenon was found to occur.

His work on the fats, in addition to supplying new methods of examination, taken as a whole, constitutes a valuable original contribution, and to regard it merely as chemical or histological would be to do it scant justice. It is an attempt to approach the inner workings of cells especially in reference to processes of oxidation, and in this respect his outlook was influenced by his earlier work on respiration. We get from time to time hints of what was in his mind in this connection, and it is a matter of regret that we did not have from him a fuller exposition.

His research work was in the two main fields which have been dealt with, but in addition he made important contributions to other subjects. His bacteriological work on typhoid fever has already been referred to, and he wrote also on Bright's disease (1912), while during the war he carried on investigations which dealt with important practical problems. Thus he took up, along with his laboratory colleagues, the question of antiseptics for war requirements, and the outcome (1915) was the introduction of the well-known "eusol," a solution of hypochlorous acid prepared by dissolving equal parts of boracic acid and chloride of lime. This came to be recognised to possess certain advantages as an antiseptic and has had an extensive use; they made also observations on its internal administration in cases of infection. A few days after the outbreak of gas warfare he recommended the use of charcoal in respirators, though it was not till long afterwards that charcoal was actually adopted. The subject of trench frost-bite also attracted his attention, and he and his colleagues carried out an experimental investigation on the effects of cold and moisture on the tissues of rabbits, from which they deduced principles for the prevention of the condition.

As a teacher of pathology, Lorrain Smith was very successful. The new methods which he developed brought home vividly to his students the facts of the subject.

He rendered manifold services of educational and administrative nature, especially in his later years. In addition to serving on the Irish University Commission, as already noted, he was appointed in 1907 a member of a Home Office Departmental Committee on humidity and ventilation in cotton weaving sheds, and in 1912 a member of a similar committee on humidity and ventilation in flax mills and linen factories. In the latter year he was asked to go as an expert to India on a similar enquiry, but was unable to do so. He was elected representative of Manchester University on the General Medical Council in 1912 and served till his appointment to the chair in Edinburgh. In 1927 he was chosen to serve in a similar capacity for Edinburgh University. In these various capacities his scientific and academic qualifications were of high value, and he gave freely both time and labour to the various questions with which he had to deal. He was one of a small group of men who were instrumental in founding the Pathological Society of Great Britain and Ireland in 1906. He



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did much to advance its interests and to bring about the success which it has attained. He was also for several years chairman of the Research Committee of the Animal Diseases Research Association of Scotland, and took a keen interest in its work.

From 1919 Lorrain Smith was Dean of the Medical Faculty of Edinburgh University, and the office was one which made considerable inroads into his time. He devoted much thought to its duties and he came to be a leading influence in guiding the educational policy of the faculty. He had a keen interest in the methods of teaching and he was the means of devising and carrying through a scheme for co-ordinating the teaching of the later years of the curriculum—a scheme which was attended by marked success.

His many services were recognised by his receiving honorary degrees from the two Universities in which he first held office—the degree of LL.D. from Queen's University, Belfast, in 1922, and that of D.Sc. from the University of Manchester in 1930, on the occasion of the jubilee celebration of its foundation.

J. S. H.
R. M.

WILLIAM DOBINSON HALLIBURTON—1860-1931.

WILLIAM DOBINSON HALLIBURTON was born in 1860. Educated at University College School, then in Gower Street, he passed naturally from it into University College, where he became a student in the Faculties of Science and Medicine, taking the B.Sc. degree of the University of London in 1879 and the M.D. in 1884. In 1892 he was elected a Fellow of the Royal College of Physicians. Amongst honours later received were honorary degrees from the Universities of Aberdeen and Toronto. He became a Fellow of the Royal Society in 1891; he served on the Council on two occasions. He took a leading part in the management of the Physiological Society, on the Committee of which he served as Chairman. He also rendered valuable service to the Physiology Committee of the Royal Society of which he was often a member and occasionally chairman, being particularly well fitted for such a part by virtue of the soundness of his judgment and the equability of his disposition. His scientific work and teaching began in 1883 when he became Sharpey Scholar and assistant to Professor Schafer in the Physiology Department of University College. His first important research was on the chemistry of muscle. He repeated and confirmed in mammals the work which had been accomplished by Professor W. Kühne in the frog, and was able to add considerably to our knowledge of the protein-constituents of muscle and the changes which occur during rigor mortis. In 1889 he was appointed Professor of Physiology in King's College in succession to Gerald Yeo. Here he continued actively to pursue biochemical research, although his work was by no means confined to chemistry but embraced several experimental investigations. The subject of biochemistry has developed enormously since the time that he first worked at it; following the development of organic chemistry, to which it is now considered properly to belong. Nevertheless Halliburton must be regarded as one of the chief pioneers in this country of its establishment as a distinct branch of biology; a position which was recognised by the Biochemical Society, which he was instrumental in founding, in electing him Honorary Member. Halliburton was fortunate in having a number of brilliant investigators associated with him at King's College, some working independently, others conjointly with himself. Amongst the latter were Sir Frederick Mott and Professor W. E. Dixon. With the former he worked on the pathological chemistry of the nervous system, with the latter on the cerebral circulation and the cerebrospinal fluid. But it was especially as a teacher that he excelled; and not only as an oral teacher but as a writer. He first made his mark in this direction by the publication of the "Essentials of Chemical Physiology" which appeared in 1893 and has passed through many editions. In 1896 he was asked by the publishers of "Kirkes' Physiology" (which had long been the popular textbook for medical students but had latterly deteriorated) to undertake the editorship. This he characteristically proceeded to do by re-writing the whole book. It was so completely transformed that the publishers decided to alter the name to "Halliburton's Physiology." Under this title it has passed through nearly twenty editions and is still probably the "best-seller" of its kind. He was also the author of a "Textbook of Chemical Physiology and Pathology" (1891) and of other works, some of which were the result of lectures given to the Royal College of Physicians (Croonian Lecture (1901), Oliver-Sharpey Lecture (1907)). Not the least valuable of his writings were his contributions on physiological chemistry to the Reports of the Chemical Society. For several years he edited 'Physiological Abstracts' for the Physiological Society and was one of the founders of the 'Quarterly Journal of Experimental Physiology.'

Halliburton endeared himself to his friends by his cheery disposition, his constant loyalty and his sympathetic nature. He suffered throughout life from a physical defect of the right arm, probably the result of infantile paralysis, but he had become so accustomed to use the left arm and hand that he was able to write rapidly and legibly, to carry out delicate adjustments in his experiments, and, in his younger days, to play games as well as most people. He was fortunate in possessing during many years a true helpmate in his wife (who survives him), and in securing a small garden-surrounded

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cottage in a secluded corner in the heart of London not too far from his work. He retired from the chair of Physiology at King's College in 1923 in consequence of a paralytic attack affecting his speech; although he largely recovered this he was no longer able to lecture or take part in public discussions. He died at Exeter on May 21 of this year, regretted by innumerable friends and by physiologists throughout the world.

E.S-S.

SHIBASÁBURO KITASATO—1852–1931.

By the death on June 13, 1931, of Baron Kitasato, the famous Japanese bacteriologist, the world has lost a man who made for himself an enduring name in the history of medical science.

He was known everywhere for three bacteriological discoveries of first-rate importance. In 1889 he cultivated, for the first time, in a pure state, the bacillus of lock-jaw, and having done so was able to clear up to a large extent the pathology of this remarkable disease. In 1890, in collaboration with Emil Behring, he made the great discovery of antitoxic serum, and in 1894 Kitasato by himself discovered *Bacillus pestis*, the cause of bubonic plague, and he worked out in a convincing manner the pathology of this ancient and deadly malady.

Shibasáburo Kitasato was born at Ogiungo, a mountain village in the prefecture of Kumamoto in the Japanese island of Kiushiu. The year of his birth has been variously stated as 1854, 1856 and 1858. The conversion of Japanese dates into English is often difficult and sometimes uncertain. In a Japanese work of the "Who's Who" type it is stated that he was born in the fifth year of Kae, and I am authoritatively informed that this corresponds to December, 1852. If this is correct Kitasato was in his seventy-ninth year at the time of his death. He was the third son of Korenubo Kitasato, a knight in the days of the feudal system in Japan.

In the obituary notice published by his pupil, Professor Mikinosuke Miyajima, in the 'British Medical Journal' (1931, vol. 2, p. 1141) we are informed that Kitasato began the study of medicine in the then recently established medical school at Kumamoto. Later, he migrated to Tokio, to the Government Medical School, which afterwards became part of the Imperial University of Tokio, and he took his medical degree there in 1883. For a time he was in the service of the Central Bureau of Public Health in the Department of the Interior. About that time Japan was rapidly coming under the influence of Western civilisation and learning and Japanese students began to be seen in European universities.

In 1885 Kitasato was sent by the Japanese Government to study bacteriology in Berlin at the Hygienisches Institut under Robert Koch, who was then nearing his zenith as a great pioneer in the study of infective diseases. Kitasato worked in Koch's institute from 1885 to 1891, and in the latter part of this time he received the title of Honorary Professor in the University of Berlin.

Robert Koch was a man of great talent, of untiring diligence and of dominating personality. By all his hundreds of pupils he was held in the highest esteem and affection notwithstanding a certain coldness which was part of his nature. The effect of Koch on Kitasato was lasting, and to the end of his life Kitasato's highest aims were that his early associations with the German investigator should be justified. Twenty years after their inspiring Berlin days when Koch visited Japan in 1908 Kitasato received him with open arms, convoyed him all over Japan and did much to make Koch's visit almost a national event in Nippon. Kitasato had his full share of the characteristic Japanese trait of the veneration of the pupil for the master and teacher, and when Koch died he took an active part in raising to his memory an appropriate monument. This "Gedenkstein" is placed at Kamakura in Sagami Bay, not far from Yokohama. It bears a Japanese inscription so full of romantic beauty and poetry that I insert a translation, as it throws a light on the personality of Shibasáburo Kitasato. The words in English are: "In the forty-second year of the reign of Meiji, in the seventh month, the great German physician and teacher Koch, professor in Berlin, came to Kamakura. He pitched his tent here on the Mountain of Spirits and morning and evening he wandered with Kitasato to enjoy the beauty of the mountain and the temple. Not long after he returned to his Western home, where he died. The owner of the mountain has joined with others in erecting this monument in commemoration of the spot that Koch loved so much. This site was chosen around which the waves of the sea dash and storm and far above gleams the snow-white peak of Fuji-no-yama, already renowned in olden days through the great battle of the famous Japanese hero, General Nitta. It was here, by Inamurasaki, that he cast his sword into the sea, and hither came the great man from across the ocean, looked across to Fuji and loved the place. Therefore let this stone stand here as an enduring memorial. Written in the first year of the reign of Taisho, in the ninth month, by Nagasaka-Shuki." Such was Kitasato's veneration for his teacher that he erected a shrine to him in the inner court of his institute, and always on May 27—the anniversary of Koch's death—he commemorated with appropriate Shinto rites the memory of the soul that had departed.

But let us return to Kitasato's scientific career. When he took his work bench in the Hygienisches Institut in Berlin in 1885 bacteriology had already established itself as a rapidly advancing science, through the discoveries of Pasteur in France and the Kreisphysikus of Wollstein, Robert Koch. In particular the simple methods of pure culture on solid, transparent media which had been devised in 1881 by Koch had already yielded such an amazing harvest of results as to inaugurate a new era in medicine and brought about a revolution in our ideas on the causation of disease. Up till 1881 the bacterial causes of disease had been rendered certain, or probable, in the case of anthrax and relapsing fever only. The new era was ushered in by Koch's own discovery of the tubercle bacillus in 1882. In the same year Loeffler and Schütz discovered the cause of glanders. In 1884 Koch discovered the cause of Asiatic cholera, Loeffler the diphtheria bacillus and Gaffky the bacillus of typhoid fever.

It was to Berlin—to Koch's Institute—where all this feverish activity was going on that Kitasato came and he was in the thick of it in 1885. After passing through the bacteriological course that Koch gave, Kitasato began to carry out independent researches and filled up several lacunæ in knowledge by laborious and accurate observations. In 1889 he published his classical work on lock-jaw. Nicolaier in 1884 had demonstrated that the introduction of particles of garden earth into house mice and other animals can induce tetanus and Rosenbach also showed that pieces of tissue from a human case of tetanus can transmit the disease. He made cultures, but could not get them pure. These cultures contained peculiar bacilli in the form of a "pin or a drumstick," and he inferred that these were probably concerned in the production of lock-jaw. It was Kitasato who proved this by successfully isolating, in a pure state and in the absence of oxygen, the drumstick bacillus, and he showed that this is capable of producing lock-jaw. In the bodies of animals dead from the disease he could not find the bacilli which he had injected, and he inferred that they must be destroyed in the body, but that before this occurred they must give rise to a chemical poison which was the cause of the undoubted involvement of the central nervous system. This tetanus poison was in fact proved shortly afterwards by Knud Faber. Kitasato's work on lock-jaw was remarkable in that he cleared up the main points in the ætiology of tetanus and nothing which he wrote on the subject in 1889 has been proved to be incorrect to-day.

Continuing his researches on tetanus Kitasato, in association with Emil Behring, made the great discovery of the antitoxic properties of blood serum, and this was announced on December 4, 1890, in a short but epoch-making paper entitled "Ueber das Zustandekommen der Diphtherie-Immunität und der Tetanus-Immunität bei Thieren." This created a sensation in the medical world, for the authors announced that they had succeeded in producing artificially an immunity against tetanus and that the immunity depended on the ability of the cell-free blood fluid to render inert the poison produced by the tetanus bacillus. Behring and Kitasato pointed out that this toxin-destroying—they named it antitoxic—property of the blood still persists after coagulation and is found in the blood serum. It was so durable that when introduced into the body of a normal animal it could enable that animal to withstand with impunity hundreds of multiples of the dose of tetanus toxin otherwise fatal in an unprotected animal. One week after the publication of Behring and Kitasato on tetanus immunity, Behring alone published one on diphtheria, and showed that similar results can be obtained in this disease. These two researches laid the basis of serum therapy and originated a new form of therapy.

Returning to Japan in 1891 with a wide bacteriological experience, Kitasato established in 1892 a private bacteriological institute on a modest scale. It was, however, the first of its kind in Japan. Through many years he fostered his institute and lived long enough to see it a flourishing school of scientific workers equal to the best that can be found in Europe. Kitasato was greatly helped by the efforts of Yuchichi Fukuzawa, one of the most progressive men of Japan, who did more than any of his contemporaries to spread in his native land a knowledge of the learning of the West, and who founded the Keiō Gijiku University in Tokio. In 1893 Kitasato's private institute was subsidised by the Japanese Government and new buildings were erected at Shiba, a suburb of Tokio.

While these events were taking place bubonic plague had broken out in China, first attracting notice in Canton early in 1894. The disease was conveyed to Hong-Kong and attacked chiefly the lower classes of Chinese, but also to some extent the British garrison. The dread of the disease was such that some 80,000 of the native population fled. Clinically the disease was identified as the pest of classical days, but nothing was known of its cause. The Japanese Government sent Professors Kitasato and Aoyama, a pathologist, to investigate the disease and in a few days Kitasato had settled the mystery by isolating the bacillus of plague and showing the cardinal part which it played.

The Japanese mission left Yokohama on June 5, 1894, and landed at Hong-Kong on the 12th. A laboratory was fixed up in a room in the Kennedy Town Hospital, one of the plague establishments. Work was begun on June 14, 1894, and in an autopsy made on that day Kitasato found numerous bacilli in the bubonic lesion, as also in the internal organs. As the examination had been made 11 hours after the patient's death, Kitasato was at first doubtful of the significance of the bacilli, but he made cultures and at the same time inoculated a mouse directly from the patient's spleen. While waiting for a result he examined the blood of a living case of severe plague and found the same type of bacilli. Cultures were made and on the following day had grown. With these cultures he inoculated mice, guinea-pigs, rabbits and pigeons. With the exception of the last all succumbed, with symptoms and lesions the same as he had found in human plague. Every day he examined fresh living cases, and from fifteen autopsies on plague patients he isolated the same bacillus every time. He gave an accurate account of the bacteriology of plague and formulated the general principles for its prevention.

In the annals of bacteriology there is no record of a micro-organism of a formidable disease being discovered in a shorter period of time, once it was investigated by a man who knew his business. A number of people tried their hand at solving the cause of plague before Kitasato stepped in, but he it was who did the successful work. Dr. James A. Lowson, of Hong-Kong, sent notes of Kitasato's discovery and also some of his preparations to England, and these were published and figured in the 'Lancet' of August 11, 1894 (p. 325). On August 25, 1894, an account of the bacteriology of plague was also published by Kitasato himself in the 'Lancet.'

I mention these dates because attempts have not been wanting to deprive Kitasato of the full credit of his discovery and this by some of his own countrymen. Claims have also been put forward on behalf of Alexandre Yersin, a surgeon of the French Colonial Service and director of the Pasteur Institute at Nha-Trang (Annam) who also went to Hong-Kong and arrived there on June 15, 1894, with a scanty equipment. By the time that Yersin had really started with his work Kitasato had got out all the fundamental facts concerning the bacteriology of bubonic plague. Yersin's paper was published in the 'Annales de l'Institut Pasteur' at the end of September, 1894 (vol. 8, p. 662).

Kitasato continued to take a deep interest in plague and wrote a good deal upon the subject in Japanese. In 1911 he went to Manchuria to study the fearsome epidemic of pneumonic plague which was then sweeping away its thousands of victims. At the International Plague Conference at Mukden he was unanimously elected President.

Kitasato visited the St. Louis Exposition in 1904 and was in England more than once. In Japan he spent his life building up the reputation of his Institute and in developing its school of workers, among whom may specially be named, Taichi Kitashima, Kiyoshi Shiga, S. Hata and Miyajima. In 1899 the Institute was taken over entirely by the Japanese Government, who greatly increased its scope until it became a large modern organisation known as the Imperial Japanese Institute for Infectious Diseases. New buildings were erected at Shirokane in the Shiba district some 3 miles south-west from the centre of Tokio, and Kitasato was made Director of the whole. This post he held for 15 years, and with great success. In 1914 the Institute was transferred to the control of the Minister of Education and in 1916 was amalgamated with the Imperial University of Tokio. Miyajima ('Brit. Med. Journal,' vol. 1, p. 1141 (1931)) tells us that this change was effected without consulting Professor Kitasato, who forthwith resigned and all his collaborators resigned with him. He then established a new private Institute erected in Shirokane Sankocho, and named the "Kitasato Institute for Infective Diseases," and there he continued his work as long as he was able.

In 1916 he was appointed a member of the House of Peers, took up political work and did a great deal to organise the Medical Practitioners' Association in Japan. In recognition of his services to science and his public spirit he was raised to the Japanese peerage as Baron Kitasato in 1924 On the day of his death he was decorated with the Grand Ribbon of the Rising Sun by H.I.M. the Emperor of Japan. He had also many foreign honours, the most treasured being his election as Foreign Member of the Royal Society on June 4, 1908. He was the only Japanese ever elected to the Royal Society. He was corresponding member of the Prussian Akademie der Wissenschaften and 'Associé Étranger' of the Académie de Médecine de France.

He founded in April, 1917, the 'Kitasato Archives of Experimental Medicine' and edited this high-class scientific periodical down to 1930, when his place was taken by his pupils, Kitashima, Shiga, Hata and Miyajima as editors.

In appearance Kitasato was typically Japanese, a short and, in his earlier days, very active man. Latterly he became somewhat corpulent, but was impressive in his dignity. His death occurred very suddenly from apoplexy. Our portrait represents him in middle life.

W. B.

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